STUDIES ON THE RHIZOBIUM TRIFOLII/ CLOVER SYMBIOSIS

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"TO MY PARENTS"

I hereby declare that this thesis has been composed by myself, and that all the work herein is my own.

Summary

As a starting point for the investigation of the role of plasmids in the Rhizobium trifolii/clover symbiosis, it was decided to search for a strain of R. trifolii having a single plasmid. Five effective strains of R. trifolii were screened for the presence of plasmids; four size-classes were detected in strain FA-6, two in TA-1, two in 204, one in P_3, while no plasmid DNA was found in 1-DL. It was impossible to say whether 1-DL lacked a plasmid or possessed (a) large plasmid(s) which fragmented during extraction. The P_3 plasmid, designated pMAM-4, had a MW of $220 \pm 0.2 \times 10^6$. To unravel the functions conferred by pMAM-4, it was thought desirable to label the plasmid with a biological marker using a technique which involved insertion of the transposable element Tn-5. By appropriate crosses between labelled strains, clones were identified which transferred kanamycin resistance at a frequency of 10^{-6} and these were presumed to carry Tn-5 on the plasmid. Further work revealed that pMAM-4 confers host-specificity and is related to the P-incompatibility group.

Symbiotic functions, i.e. nodulation and fixation, were eliminated from several clones of R. trifolii 204 incubated at an elevated temperature; a possible correlation with plasmid-loss was suggested. Other symbiotic-defective mutants, of R. trifolii P_3, were isolated following transposon mutagenesis. However, mapping experiments using R. leguminosarum as the recipients

showed multiple sites of insertion of the transposon into the chromosome which appears to preclude this approach as a method for mapping symbiosis-genes in R. trifolii P_3.

The relative nodulating success with Trifolium repens cv. Huia of three genetically-marked strains of R. trifolii, two from the U.K., i.e. (MD-1b) and (MD-1c) and one from Iraq, having a high maximum growth temperature, i.e. (MD-1a), was assessed. Inocula of paired competitors were supplied in a 1:1 ratio at different temperatures. Using aseptic test-tube culture, the two U.K. strains competed equally for nodulation at 20°C. Their competitive behaviour was very evident at 15°C when combined separately with (MD-1a); although at 25°C their competitive advantage over the Iraqi strain was reduced. A similar effect of temperature was noted when the number of mixedly-infected nodules at 15°C and 25°C were compared. The ratios of the strains in the mixedly-infected nodules generally deviated from a 1:1 ratio. Thus (MD-1c) always formed the majority of the population when present with either (MD-1b) or (MD-1a). An approach for the exploitation of competition in Rhizobium was discussed.

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CHAPTER I

"GENERAL INTRODUCTION"

IMPORTANCE OF BIOLOGICAL FIXATION OF NITROGEN

Although an abundant supply of nitrogen is present in its elemental form in the atmosphere (approximately 80% by volume of the air is nitrogen), it can enter biological systems only when it has been "fixed" or combined with certain other elements, such as hydrogen or oxygen (Brill, 1977). Fixation of nitrogen occurs via three major routes (Postgate, 1980): spontaneous, i.e. the formation of nitrogen oxides in the atmosphere as the result of combustion, electric discharges and UV-irradiation; industrial, almost exclusively by the Haber-Bosch process, i.e. the catalytic reduction of N_2 to NH_3 with H_2; and biological fixation, i.e. the conversion of N_2 to NH_4^+ and thence to cell material by prokaryotes. Quantitative estimates of the contributions of the various routes differ greatly in their precision (Burris, 1977). Burns and Hardy (1975) estimated that industrial fixation contributes 40×10^6 tonnes of nitrogen, other abiological processes 45×10^6 tonnes and biological fixation 175×10^6 tonnes, per annum. These figures, coupled with the disadvantages of the Haber-Bosch process such as the expense of manufacture and the cost of transport and distribution (Pimentel, 1976), serve to emphasise the paramount importance of biologically-fixed nitrogen over chemically-fixed nitrogen (see review by Evans and Barber, 1977). Biological nitrogen fixation is restricted to certain micro-organisms. It was the demonstration that the ability of a microbe to fix nitrogen is correlated with its ability to reduce

acetylene to ethylene (Dilworth, 1966), that has revolutionized the list of accepted nitrogen-fixing systems during the last two decades (see review by Dalton, 1974; Yates, 1977). Some of the organisms can fix nitrogen while in the free-living state, e.g. Azotobacter and blue green algae (Cyanobacteria), while others do so only when associated with plants, e.g. Rhizobium-legume symbiosis. Nitrogen fixation by Spirillum lipoferum in association with the roots of Digitaria decumbens is still a matter for investigation (Döbereiner and Day, 1976). Some rhizobia have been shown to be capable of aerobic nitrogen fixation ex planta if the oxygen tension is low enough (Keister, 1975; Kurz and La Rue, 1975; McComb et al., 1975; Pagan et al., 1975). All the above mentioned prokaryotes share a unique enzyme system, nitrogenase (Eady and Postgate, 1974), which reduces N_2 to NH_4^+.

Among the various biological systems that are able to fix atmospheric nitrogen, the symbiosis of Rhizobium with Leguminoseae contributes a significant amount of fixed nitrogen to the ecosystem and to food production (Table 1-1). The leguminous crops are known for their ability to enrich with fixed nitrogen the soil in which they are grown. This beneficial effect was realized by the ancient Chinese, Greeks and Romans (Fred, Baldwin and McCoy, 1932), and led to a widespread use of crop rotation. Unfortunately, legumes form merely 13% of world food production,

Table 1-1: Estimates of annual biological nitrogen fixation on earth* (figures in parentheses indicate alternative values)

Systems	KgN_2 fixed (ha x yr)	Metric tons/yr x 10^{-6}
Legumes	140 (80)	35 (20)
Non legumes	35	9
Permanent grassland	15 (8)	45 (24)
Forest and woodland	10 (5)	40 (20)
Unused land	2	10
Total land		139 (83)
Sea	1 (0.5)	36 (18)
Total		175 (101)

* From Subba Rao (1980)

while 50% is formed by cereals (Hardy, 1976). The cereal grain crops produce their elevated yields in response to increased fertilizer application. Thus we are faced with one of the greatest challenges of our time, the supply of N-fertilizer. This has led to an increasing world-wide effort to enhance biological production of the nitrogen fertilizer as the process of choice in the future. The prospects for the exploitation of the biological process have been the subject of numerous publications, e.g. Hardy (1976, 1977), Burris (1977), Postgate (1977), Wittwer (1977), Gutschick (1978), Andersen et al. (1980). Possibilities range from those likely to be effective in the short term, e.g. expanded use of grass and woody symbioses, through medium term, e.g. genetic manipulation of the appropriate bacteria to yield effective symbiosis, to long term, e.g. development of new nitrogen fixing systems by somatic hybridization of plants. In recent years, steps towards the achievement of these applied goals have been started (Johnston et al., 1978a; Davey and Cocking, 1980).

AIMS OF THIS STUDY

The aims of this study were, firstly, to elucidate the role of plasmids in nitrogen fixation in Rhizobium trifolii. To achieve this, several lines were followed. These were: screening effective strains of R. trifolii for the presence of single-size class

plasmid DNA; elimination of the plasmid from R. trifolii and assessing the symbiotic properties of the plasmidless strains; transfer of the plasmid to other species of Rhizobium and assessment of the symbiotic properties acquired (since plasmids in R. trifolii are assumed to be cryptic, biological labelling is a prerequisite for this step); and application of efficient mutagenic procedures for the isolation of symbiotically-defective mutants and mapping of the mutations generated. Secondly, to evaluate the competitive ability of strains of R. trifolii, in nodulating clover, from different geographical origins, taking the temperature (an environmental limiting factor for the symbiotic process) into consideration. The practical use of naturally-selected or genetically-altered superior nitrogen-fixing Rhizobium strains is hampered by an ignorance of how to improve their competitiveness for nodulation compared with that of indigenous strains already in the soil. Other problems include the lack of understanding and present inability to solve the ecological problems such strains encounter in the fiercely competitive situation in soil and on the root.

ESTABLISHMENT OF AN EFFECTIVE RHIZOBIUM-LEGUME SYMBIOSIS

The process that begins on the root surface and culminates in the establishment of an effective N_2-fixing nodule is a multi-stage sequence of interdependent steps. The first step, recognition,

appears to involve the specific-binding of exo- or lipopoly-saccharides of the rhizobia to lectins in a manner comparable to that of an antigen-antibody reaction (Beringer, 1978). It has been suggested that the initial attachment is cemented by cellulose fibrils of rhizobial origin (Napoli, Dazzo and Hubbell, 1975). Infection of the plant is the next step. In the normal infection sequence, the root hair curls in response to the dense rhizobial population which aggregates at its surface. Possible involvement of gibberellin has been suggested (Dixon, 1969). Pectinase-production by the rhizobia may soften the root-hair cell wall (Hubbell, Morales and Umali-Gracia, 1978) which with the plasma-lemma invaginates into the root hair, and subsequently the root, forming an "infection thread" containing rhizobia. Growth of the infection thread may be stimulated by the auxin indole acetic acid (IAA) produced by rhizobia (Dart, 1975). The latter also produce cytokinins which may diffuse into the root cortex and stimulate cell division leading to formation of the nodule into which the infection thread grows (Syono, Newcomb and Torrey, 1976). The rhizobia are "budded off" from the tip of the infection thread in vesicles surrounded by plasmalemma, into the nodule cells, where they differentiate into spheroplast-like organisms called bacteroids which are the site of nitrogen fixation (Jordan, 1962). In an effective symbiosis, leghaemoglobin is produced, the haem moiety by the bacteria and the globin moiety by the plant (Cutting and Schulman, 1971).

The nitrogenase enzyme complex is synthesized and nitrogen is fixed. Further understanding of the symbiotic process requires both an analysis of the genetic determinants controlling the intrinsic properties of the partners and their mutual compatibility, as well as a study of the influence exercised by the environment on each partner separately and on their interaction.

PROKARYOTIC DETERMINANTS

Three gross symbiotic phenotypes and their genetic designation have been classically distinguished: infectivity (Inf^+), which is the ability of a strain to induce nodule formation; effectiveness (Eff^+), generally applied to a Rhizobium strain which can initiate the development of nodules on its host and can proceed through the several stages required to form the nitrogen-fixing bacteroid-containing tissue in the nodule; and host specificity (Hsp^+), which is the ability of a strain to nodulate a specific host. Most reports of the symbiosis are in such general terms. However, a more analytical scheme for nomenclature has been initiated by Dénarié and Truchet (1976). They categorized the genes controlling effectiveness into three groups: those affecting Eff^+ but having a characteristic observable in vitro were represented by the symbol corresponding to the in vitro phenotype, e.g. leu, ade; those affecting Eff^+ but having no known in vitro phenotypic characteristic were called eff and genes controlling nitrogenase synthesis were

designated nif. The shorthand name nif was generalized by Postgate (1978) to describe the genetic information enabling bacteria to fix nitrogen. He proposed that any mutation in the nif genes which leads to the failure of its expression should be given a minus sign (nif$^-$), and for those which have not been positively identified as in nif, the term Nif (capital letter and no italics) should be used. As more information on the three major recognizable stages became available, a framework was formulated by Vincent (1980) within which the genotypic designation of the detailed stages, grouped to conform with three steps, were conveniently coded (see Table 1-2). However, Brewin, Johnston and Beringer (1980) proposed the designations Nod$^-$ (i.e. not producing visible root nodules) and Fix$^-$ (unable to fix N_2 or reduce acetylene, within nodules) on the basis that these designations are more easily ascribed. Indeed a Nod$^-$ mutant might be blocked at the stage of nodule meristem initiation and this mutant would thus be "infective" in the sense that it still forms infection threads. For these reasons the designations of Brewin et al. (1980) will be used, as far as possible, throughout this thesis.

LOCATION OF SYMBIOTIC GENES IN RHIZOBIUM

Many properties not essential for cell viability are determined by extrachromosomal genes. Examples include bacteriocin production and resistance (Meynell, 1973), antibiotic production

Table 1-2: Analysis of symbiotic sequence*

Stage	Abridged description	Phenotypic code
I. Pre-infection		
1. Multiplication on root-surface (rhizoplane)	Root colonization	Roc
2. Attachment to root surface	Root adhesion	Roa
3. Branching of root hairs	Hair branching	Hab
4. "Marked" curling of root hairs	Hair curling	Hac
II. Infection and Nodule Formation		
5. Formation of infection thread	Infection	Inf
6. Development of poly-ploid (disomatic) meristem; nodule development and differentiation	Nodule initiation	Noi
7. "Intracellular" release of rhizobia from infection thread	Bacterial release	Bar
8. "Intracellular" multi-plication of rhizobia and development of full bacteroid form	Bacteroid development	Bad
III. Nodule Function		
9. Reduction of N_2 to NH_4^+ (nitrogenase)	Nitrogen fixation	Nif
10. Complementary bio-chemical and physio-logical functions	Complementary functions	Cof
11. Persistence of nodule function	Nodule persistence	Nop

*From Vincent (1980)

in Streptomyces (Kirby, Wright and Hopwood, 1975), degradation and utilization of certain exotic substrates in Pseudomonas (Chakrabarty, 1976) and oncogenicity conferred on Agrobacterium tumefaciens by the so-called "Ti" (tumour inducing) plasmids (Van Larebeke et al., 1974; Watson et al., 1975). "Ti" plasmids are large plasmids of MW ranging from about 100 to 160 x 10^6 (Zaenen et al., 1974; Sciaky, Montoya and Chilton, 1978). They carry genes that determine whether or not tumours induced on their hosts will contain unusual amino acids such as octopine or nopaline (Schell and Van Montagu, 1977). They can be introduced into strains lacking them by mobilization with the promiscuous plasmid RP_4 (Chilton et al., 1976). Recently, it was found that "Ti" plasmids can act as conjugative plasmids themselves. However, conjugation only takes place when donor and recipient bacteria are incubated on a solid minimal medium containing for example octopine for octopine-coding Ti plasmids (Genetello et al., 1977; Kerr, Manigault and Tempé, 1977; Petit et al., 1978). Mutants of A. tumefaciens can be isolated that carry plasmids derepressed for transfer (Hooykaas, Roobol and Schilperoort, 1979). The properties of Agrobacterium may be relevant to those of Rhizobium since both genera are able to induce cell multiplication in host plants. Taxonomic similarities (Graham, 1976) were strengthened by the demonstration that the "Ti" plasmid could be transferred from A. tumefaciens into R. trifolii. Transconjugants were able

to induce tumours but were still able to form effective nodules on the legume host. The type of induced tumours and the regulation of "Ti" transfer were similar in both bacteria, confirming their close relationship (Hooykaas et al., 1977). A bacterial strain has recently been reported that can induce both nodule formation on clover and crown gall on a suitable host (Skotnickie and Rolfe, 1978). Based on the resemblance mentioned above and certain additional evidence which supports the suggestion that host specificity genes may be extrachromosomal (see later), a hypothesis is presented here, that plasmids in Rhizobium play an essential role in establishing symbiosis on legumes, as the "Ti" plasmids do in the formation of crown gall by A. tumefaciens.

INVOLVEMENT OF PLASMIDS IN SYMBIOSIS IN RHIZOBIUM

Plasmids have been detected in different species and strains of Rhizobium using DNA sedimentation profiles in sucrose or caesium chloride (CsCl)-ethidium bromide (EtBr) gradients (Klein et al., 1975; Tshitenge et al., 1975; Zurkowski and Lorkiewicz, 1976; Olivares, Montoya and Palomares, 1977). The commonly employed cleared lysate procedure (Clewell and Helinski, 1969) allowed the isolation of plasmids of low molecular weight. Thus strains of R. trifolii were reported to carry plasmids of 28×10^6 (Dunican, O'Gara and Tierney, 1976) and 5.5×10^6 (Kowalczuk and Lorkiewicz, 1977). However, Nuti et al. (1977) pointed out that

large plasmids in Rhizobium must have been overlooked in earlier studies, since the initial step in that procedure involved the removal of chromosomal DNA as part of a membrane complex and large plasmids would have behaved as if they were chromosomal. This phenomenon has been encountered with other large plasmids (Palchaudhuri and Chakrabarty, 1976). Following the method which was devised for the isolation of large covalently-closed circular (CCC) DNA molecules from A. tumefaciens (Zaenen et al., 1974; Ledeboer et al., 1976; Currier and Nester, 1976), Nuti et al. (1977) detected large plasmids in strains of R. leguminosarum, R. trifolii, R. japonicum and Rhizobium "cow pea". As estimated by renaturation kinetics, the molecular weights of these plasmids were in the range of 70 to 400 x 10^6. Similar observations were made by Casse et al. (1979) who identified plasmids in 25 effective R. meliloti strains: plasmids of 90 - 200 x 10^6MW, assessed by relative electrophoretic mobility on agarose gels and electron microscopy, were found in 22 strains, eight of which carried more than one plasmid size. Differences in plasmid number and molecular weight exhibited by strains of R. japonicum from different geographical origins led Gross, Vivader and Klucas (1979) to consider the plasmid profiles to be a rapid and reliable method for strain distinction. The suggestion that these plasmids are active participants in symbiosis has been tested in two ways:

(a) Curing of plasmid DNA:

Chemicals, such as acridines used at sub-bacteriostatic concentrations, can eliminate some plasmids from their hosts, a phenomenon known as "curing". The response of infectiveness, using acridine orange and sodium dodecyl sulphate as curing agents and mitomycin C as an inducing one, was studied by Higashi (1967); Parijskaya (1973); Zurkowski, Hoffman and Lorkiewicz (1973). After treatment of infective Rhizobium with the curing chemicals, these authors found a sharply decreasing ability of bacteria to form nodules on the corresponding legume root. Dunican and Cannon (1971) correlated effectiveness with the presence of a plasmid in R. trifolii since a change of viomycin sensitivity and resistance occurred after treatment of bacteria with a curing agent. Effectiveness has also been linked to an indigenous resistance plasmid (R-plasmid) in R. trifolii (Dunican, O'Gara and Tierney, 1976). Though loss of the plasmid and, concurrently of effectiveness, was claimed by the authors to be the result of treatment with EtBr, the strain used was already ineffective. Moreover, loss of a property after treatment with a curing agent could be due to any one of a number of effects especially when it is known that a characteristic such as infectivity can be lost at high frequency as the result of a pleiotropic mutation, e.g. one leading to auxotrophy (Sherrer and Dénarié, 1971) or antimetabolite resistance (Schwinghamer, 1968). This makes it rather difficult

to interpret these reports. However, following incubation at 35°C for seven days, Zurkowski and Lorkiewicz (1978) isolated non-nodulating mutants of R. trifolii and suggested that these were the result of the loss of a plasmid. Similarly, Casse et al. (1979), following heat treatment of the wild type, obtained a non-nodulating mutant of R. leguminosarum which had been cured of the smallest of the three plasmids contained in the parental strain. It would appear, therefore, that nodulation genes are plasmid-borne, at least in some strains of Rhizobium.

(b) Transfer of plasmid DNA:

In only three early cases have there been reports of the transfer of plasmids carrying symbiotic genes. Higashi (1967) reported transfer of the ability to nodulate clover from R. trifolii to R. phaseoli. He proposed a plasmid nature for this property, i.e. host specificity, since transfer was apparently unassociated with the transfer of other chromosomal markers. However, this work is rather rudimentary as the bacteria used did not carry other genetic markers. Dunican and Tierney (1974) presented evidence for the transfer of nitrogen-fixing genes from R. trifolii to Klebsiella aerogenes using a derepressed R-factor, R1-19drd. Following the same protocol, Dunican, O'Gara and Tierney (1976) claimed a plasmid model for effectiveness on the basis that transfer of nitrogen-fixing genes from R. trifolii to K. aerogenes occurred at a frequency higher than that expected for chromosomally located

markers. However, a selectable marker on a plasmid would provide the best way of following transfer of the plasmid and to assess its presence or absence in a cell. Innate ones such as antibiotic resistance (Cole and Elkan, 1973), induction of polygalacturonase-production (Palomares, Montoya and Olivares, 1978), phage sensitivity (Corral, Montoya and Olivares, 1978) and bacteriocin production (Beringer, 1976) were all claimed to be linked to the resident plasmids in Rhizobium. Recently, the conjugative ability of a bacteriocinogenic plasmid has been demonstrated (Johnston et al., 1978a). The insertion into this plasmid of a transposon coding for kanamycin resistance, i.e. Tn-5 (the vector used and the potential of using transposons in Rhizobium will be discussed later), provided the first definitive evidence for the participation of plasmids in host specificity and nodulation. Johnston et al. (1978a) followed the transfer of Tn-5 into different species of Rhizobium and into a non-nodulating strain of R. leguminosarum and showed that all had acquired the ability to nodulate peas, the specific host of R. leguminosarum. But the carrying by a strain of more than one plasmid led to the question of diagnosing the one(s) involved in the symbiotic process. However, in view of the reports implicating plasmids as the site of symbiotic genes, it is relevant to ask whether any symbiotic gene at all is harboured on the chromosome. In several interspecific crosses, involving transfer piecemeal of about 50% of the chromosome, Johnston and Beringer (1977) found no evidence for the

transfer of host specificity from R. phaseoli and R. trifolii to R. leguminosarum. These negative results provided circumstantial evidence that the genes determining host range are extrachromosomal. A classification of symbiotic genes on the basis of their location could be obtained by mapping. However, the prerequisite for any genetic analysis is the isolation of mutants and the development of a system for gene transfer.

INDUCTION OF MUTATION IN RHIZOBIUM

The induction of mutations in rhizobia has been achieved by employing a variety of mutagenic agents, those most commonly used being N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (Schwinghamer, 1969; Beringer, 1974; Kondorosi et al., 1977), ultraviolet light (UV) (Gupta and Kleczkowska, 1962; Kowalski, 1970) and ethyl methanesulphonate (EMS) (Schwinghamer, 1969; Kaushik and Venkataraman, 1972; Meade and Signer, 1977). The utility of such mutagenic treatments for the production of symbiotic mutants is questionable (for review and criticism, see Cunningham, 1979) as they require every surviving clone to be assessed for its symbiotic ability. This problem may be relieved in part by employing transposons which can mark the mutated gene with a selectable phenotype. Transposons are discrete sequences of DNA, incapable of self-replication and can insert into DNA replicons, such as the chromosome or the plasmid independent of

host recombinational ability (Kleckner, Roth and Botstein, 1977). Insertion can be random and, where it occurs in a gene, leads to a non-leaky polar mutation. Some transposons code for drug resistance, e.g. Tn-5 (Berg et al., 1975). However, in order to introduce Tn-5 into Rhizobium, Beringer et al. (1978b) constructed a P1-group plasmid called pJB4JI. This plasmid is not replicated in Rhizobium due to insertion within it of the phage Mu genome. Using this vector for Tn-5 insertion, auxotrophic mutants were isolated in R. leguminosarum, R. trifolii and R. phaseoli (Beringer et al., 1978b). However, transposon-induced mutations can be located by mapping the position of the antibiotic resistance carried on the transposon.

GENE MAPPING

(a) Transformation:

There have been reports of transformation in several Rhizobium species (Balassa, 1963; Raina and Modi, 1972). However, most transformation experiments were carried out between strains differing only in the marker being tested (for review and criticism, see Beringer, 1973) and no transformation system is available for fine-scale genetic mapping.

(b) Transduction:

Most studies on transduction have involved R. meliloti (Kowalski, 1970, 1974). Svab et al. (1978) described a specialized

transducing phage for a cysteine marker on the chromosome of a R. meliloti strain. Generalized transduction has also been demonstrated in R. meliloti (Casadesus and Olivares, 1979a, 1979b) and in R. leguminosarum (Buchanan-Wollaston, 1979). The bacteriophage described by Buchanan-Wollaston (1979) could transduce a number of markers from R. leguminosarum to R. trifolii but no transduction was detected in the reverse direction. It has also been used to map closely-linked markers on the R. leguminosarum chromosome (Johnston et al., 1978a).

(c) Conjugation:

Of the three major forms of gene transfer in bacteria: conjugation, transformation and transduction, conjugation is potentially the most useful for preliminary studies of gene linkage because relatively large fragments of the genome may be transferred.

Rhizobium was shown to be capable of conjugal gene transfer, as witnessed by its ability to receive and donate the wide host range, P-group R-factor RP4 (Datta and Hedges, 1972; Olsen and Shipley, 1973; Beringer, 1974). P-group R-factors are capable of mobilizing chromosomal genes in Pseudomonas aeruginosa (Haas and Holloway, 1976) and Acinetobacter calcoaceticus (Towner and Vivian, 1976) as well as R. meliloti (Boucher et al., 1977). Indeed, circular linkage maps of R. meliloti have been constructed using R68.45 (Kondorosi et al., 1977; Casadesus and Olivares, 1979a)

and RP4 (Meade and Signer, 1977) and of R. leguminosarum using R68.45 (Beringer, Hoggan and Johnston, 1978). Kondorosi et al. (1980) compared the maps of R. leguminosarum and R. meliloti and showed that the map lengths and the positions of many mutations for particular nutritional requirements were very similar. However, features of the behaviour of R68.45-mediated recombination in Rhizobium were summarized by Beringer, Brewin and Johnston (1980). In all cases very large segments of the donor chromosome are transferred from donor to recipient, and within the recipient these large sections replace homologous chromosomal segments with very little internal recombination. Another interesting finding is that the frequencies of recombination in crosses between R. leguminosarum and R. trifolii or R. phaseoli are about the same as between derivatives of the same isolate of R. leguminosarum (Johnston and Beringer, 1977), implying substantial chromosome homology between these three species. However, in crosses between R. leguminosarum and R. meliloti, the frequency of recombination of chromosomal markers is much reduced and most of the recombinants are R-primes (Johnston, Setchell and Beringer, 1978c). R-primes allow Rhizobium DNA to be introduced into other hosts such as E. coli or P. aeruginosa, although it appears that Rhizobium genes are not normally expressed in the former (Johnston, Bibb and Beringer, 1978b).

RELEVANCE OF RECOMBINATION STUDIES TO SYMBIOSIS

Most of the fundamental research on gene transfer systems in Rhizobium has involved auxotrophic and antibiotic-resistant mutants. Of what benefit are such studies to an understanding of symbiosis?

To date, commercial inoculum strains have been selected from unmodified field isolates. The fact emerges from recombination studies that gene transfer can occur between different Rhizobium species. This should provide a solid basis for constructing strains possessing the best attributes of different parental strains. Such attributes could include high levels of effectiveness, tolerance to extreme environment and competitiveness. However, the contemplation of a Rhizobium breeding programme should be preceded by an analysis of the rules that might govern the aforementioned desirable characteristics.

COMPETITION IN RHIZOBIUM

In natural environments, competition, e.g. the rivalry for limiting nutrients or other common needs, is one among a number of relationships that can exist between individual microbial species. Rhizobium, being a symbiont, must compete in the soil as a free-living bacterium before the onset of the symbiotic process. The survival of Rhizobium in the complexity of the soil system is affected by non-biological factors such as pH (Vincent, 1958b;

Holding and King, 1963; Jones, 1963; Munns, 1968), temperature (Vincent, 1958a; Marshall, 1957), desiccation (Hamdi, 1971), soil type (Johnson and Means, 1963; Damirgi, Frederick and Andersen, 1967) and soil moisture (Sherwood and Masterson, 1974). Interactions of rhizobia with indigenous soil micro-organisms exert a considerable influence on the ability of rhizobia to become established in the soil (Damirgi and Johnson, 1966; Hattingh and Louw, 1969). Having survived all adverse factors and multiplied, rhizobia then have to infect the host, which again requires the right conditions and successful inter-strain competition with other rhizobia. In general, a nodule is formed by only one strain of Rhizobium (Johnson and Means, 1963; Dudman and Brockwell, 1968), although different nodules on the same plant could contain different strains (Vincent and Waters, 1953). However, in tube-culture experiments, 10% of clover and soybean nodules may contain two strains of a Rhizobium species (Skrdleta, 1970; Marques Pinto, Yao and Vincent, 1974). Johnston and Beringer (1976) showed, in laboratory experiments, that the presence of R. leguminosarum can allow R. trifolii and R. phaseoli to enter the nodules of pea, and van Rensburg and Strijdom (1972) reported that a large proportion of soybean-root nodules contained an unidentified fast-growing contaminant as well as R. japonicum. Studies of Rhizobium competition necessitate the development of methods for strain distinction.

STRAIN IDENTIFICATION IN RHIZOBIUM

Recognition of Rhizobium strains has depended mainly on morphological, serological, biochemical and genetic tests. Characteristics based on morphological grounds such as distinctive nodule form (Vincent, 1970), colour (Cloonan, 1963) or unusual colony morphology (Marques Pinto, Yao and Vincent, 1974) have been used, though none is of general application but can be a useful secondary criterion. Identification of rhizobia re-isolated from nodules has, in the past, been based almost exclusively on serological methods (Read, 1953; Marshall, 1956; Date and Decker, 1965; Schmidt, Bankole and Bohlool, 1968). Serology permits the persistence of inoculant strains to be determined with considerable precision provided that appropriate controls are used. Many studies based the differentiation between Rhizobium strains on a large range of biochemical and metabolic tests. These include such properties as vitamin requirements (Graham, 1963) and utilization of carbohydrates and Krebs Cycle intermediates (George and Ettinger, 1941; Graham, 1964 ; Graham and Parker, 1969). The genetic aspect involves mutation to auxotrophy and resistance to antibiotics (Johnston and Beringer, 1975). These mutants should be free of pleiotropic effects involving symbiotic properties (Pankhurst, 1977). Recently, enzyme polymorphism has been suggested as a recognition test (Myron, McAdam and Portlock, 1978).

COMPETITION STUDIES; PAST AND PRESENT

Competition between strains of rhizobia occurs in broth and peat (Marshall, 1956; Vincent, Thompson and Donovan, 1962). Brockwell and Dudman (1968) showed that competition for available infection sites on roots also occurs. It can take place between indigenous soil populations (Johnston and Means, 1963; Ham, Frederich and Anderson, 1971), among introduced strains (Caldwell, 1969) and between introduced and indigenous soil populations (Abel and Erdman, 1964). Roughley, Blowes and Herridge (1976) investigated the numbers of naturalized populations of R. trifolii at five sites in Australia and reported a range from no detectable rhizobia to 4×10^6 rhizobia/g soil. There were marked differences in competitive ability among the introduced strains and these differences were modified by the host cultivar and the site. At locations where rhizobia were abundant at sowing, they formed most of the nodules regardless of the inoculum used. However, the current emphasis on redeveloping soils already colonized by rhizobia poses the necessity of clarifying the parameters responsible for competitive advantage.

THE BASIS OF COMPETITIVE SUCCESS

In looking for a property that might explain competitive success in nodule formation, attention was first paid to the relative growth rates of competing strains (Nicol and Thornton, 1941). However,

there were too few comparisons to justify the use of this property to predict events on the root surface leading to nodulation. In later experiments, relative growth rate did not consistently relate to success in nodulation (Vincent and Waters, 1953). Properties such as bacteriocinogeny and lysogeny can markedly alter the composition of mixed strain inoculants in broth and peat culture when producer and sensitive strains are present in the mixture (Schwinghamer and Brockwell, 1978). Recently, Evans, Barnet and Vincent (1979a, 1979b) studied the effect of a virulent rhizobiophage on the populations of R. trifolii in the rhizoplane and on the relative competitiveness of pairs of R. trifolii strains. The presence of the rhizobiophage reduced the rhizoplane population of a susceptible strain of R. trifolii and in competition favoured resistant or partially resistant strains which were otherwise less able to form nodules. It has not been possible to attribute relative competitiveness of paired strains to any single feature of their symbiotic capacity, e.g. the N_2-fixing effectiveness with a particular host. There have been many cases where the effective N_2-fixing strain appeared to have an advantage over an ineffective competitor (Robinson, 1969; Marques Pinto, Yao and Vincent, 1974; Labandera and Vincent, 1975). On the other hand, there have been some instances where the less effective strain was the better competitor (Johnston and Beringer, 1976), and marked differences in nodulating competitiveness have also been found among fully effective strains (Franco and Vincent, 1976).

These conflicting results were generally explained by assuming that a "greater compatibility with the host" played a dominant role in determining which strains form nodules. Interactions between the competing strains and the host were noted by Caldwell and Vest (1968) who showed that closely-related soybean genotypes had similar distributions of rhizobia in their nodules. Roughley, Blowes and Herridge (1976) reported that R. trifolii strain W495 was outstanding on Woogenllup cultivar as compared to Mt. Barker cultivar. In all these cases, the nature of the "extra" compatibility was not apparent.

Studies carried out over the last 40 years on the factors which may control competitiveness have failed to produce improved experimental design and have also failed to produce consistent interpretations of results such that no coherent picture of competitiveness has evolved. What appears to be needed is an altered approach to the study of competitiveness. While not claiming that the results on competitiveness in this thesis have been the breakthrough, nevertheless ideas generated in the course of these studies will be presented in the discussion.

CHAPTER II

"GENERAL MATERIALS AND METHODS"

BACTERIAL STRAINS AND PLASMIDS USED

The strains of bacteria used are recorded in Tables 2-1 to 2-3, together with the relevant details of their genotype or phenotype and source. The plasmids used are listed in Table 2-4. Some of them were transferred to hosts other than those in which they were received.

PHAGES

Two donor specific phages PRR1 and PR4 used during the course of this study were supplied by Dr. N.S. Willets (Department of Molecular Biology, University of Edinburgh). A rhizobiophage, Rt1, supplied by Dr. D. Walton was used as well.

PLANTS

All experiments were carried out with Trifolium repens cv. Huia.

MEDIA

The features common to the preparation of most media will be listed below and assumed in all cases unless indicated otherwise:

(a) Distilled water was used throughout.

(b) Media were solidified as required with $15gl^{-1}$ Difco Bacto Agar (Difco Laboratories, Detroit, Michigan).

(c) Agar media were made in 1 litre amounts in Roux flasks. Melted media were poured into plastic petri dishes (Sterilin,

Table 2-1: Strains of Rhizobium trifolii used/isolated

Strain designation	Relevant phenotype or genotype (a) (b)	Source
FA-6	wild type	Prof. J. Holding (c)
TA-1	wild type	"
1-DL	wild type	"
MD-1c	str-1 (derived from 1-DL)	this thesis
P_3	wild type	Prof. J. Holding
Derivatives of P_3		
MD-1b	rif-1	this thesis
MD-2b,a to t	rif-1 (pMAM4::Tn-5)	"
MD-3b to 7b	rif-1 (Fix^-::Tn-5)	"
MD-8b	MD-3b (pJB_3JI)	"
MD-9b	MD-4b (pJB_3JI)	"
MD-10b	MD-5b (pJB_3JI)	"
MD-11b	MD-6b (pJB_3JI)	"
MD-12b	MD-7b (pJB_3JI)	"
204	wild type	Dr. Y. A. Hamdi (d)
Derivatives of 204		
MD-1a	spc-1	this thesis
MD-2a to 10a	spc-1 Nod^-	"
MD-11a to 15a	spc-1 Fix^-	"

(a) Abbreviations: Antibiotic resistance, rif - rifampicin; str - streptomycin; spc - spectinomycin; others: Fix^-, Nod^- - symbiotically defective.

(b) Plasmids carried by these strains are in parentheses.

(c) Department of Agriculture and Food Microbiology, Queen's University of Belfast, Belfast, Northern Ireland.

(d) Institute for Applied Research on Natural Resources, Scientific Research Foundation, Jadiriyah, Baghdad, Iraq.

Table 2-2: Strains of Rhizobium leguminosarum used/isolated

Strain designation	Relevant genotype (a) (b)	Map section (c)	Source
1860	met-12 rib-2 str-69	1	Dr J.E. Beringer (d)
1433	ade-27 rib-2 str-69	2	"
1628	ura-14 ade-27 str-75	3	"
1062	ura-14 trp-16 str-86	4	"
1056	ura-14 met-14 str-84	5	"
1629	cys-8 ura-14 ade-88 str-69	8	"
1840	cys-8 ura-14 str-69	9	"
DC-11	ser-2 met/cys-19 str-1	6	Dr D.A. Walton (e)
DC-21	ser-2 ade-88 str-2	7	"
Derivatives of 1056 MD-1d, a to t	ura-14 met-14 str-84 (pMAM-4::Tn-5)		this thesis

(a) Abbreviations: growth requirements - rib - riboflavin; met - methionine; ade - adenine; ura - uracil; trp - tryptophan; cys - cysteine.
(b) Plasmids carried by these strains are in parenthesis.
(c) See Fig. 2-1.
(d) Rothamsted Experimental Station, Harpenden, Herts, AL5 2JQ.
(e) Department of Genetics, University of Birmingham.

Table 2-3: Other species used

Strain designation	Relevant genotype (a)	Source
Escherichia coli		
1830	*pro met nal* (pJB4JI)	Dr J. E. Beringer
1843	*pro met nal* (pJB3JI)	"
J5-3 (RP_4)	*pro met nal* (RP_4)	Dr B. E. Moseley (b)
Pseudomonas aeruginosa		
PAO-1670	*ade leu rif Cm*	Dr N. S. Willets
(MD-1e)	*ade leu rif Cm* (RP_4)	this thesis

(a) Abbreviations: Antibiotic-resistance - *nal* - nalidixic acid; Growth requirements - *pro* - proline; *leu* - leucine.

(b) Department of Microbiology, University of Edinburgh, West Mains Road, Edinburgh EH9 3JG.

Table 2-4: Plasmids used in this study

Plasmid	Compatibility group	Selectable markers (a)	Source
pJB_4JI	P1	Gm Spc Sm::Mu::Tn-5	Dr. J.E. Beringer
pJB_3JI	P1	Ap Tc	Dr. J.E. Beringer
RP_4	P1	Ap Km Tc	Dr. B.E.B. Moseley

(a) Abbreviations: Antibiotic resistances: Ap - Ampicillin; Km - Kanamycin; Tc - Tetracycline; Gm - Gentamycin; Spc - Spectinomycin; Sm - Streptomycin.

Others: Mu - phage Mu; Tn-5 - transposon coding for kanamycin resistance.

Teddington, Middlesex). All plates were dried before use.

(d) Liquid media were made up in 200 ml in medical flats.

(e) Sterilization of media, filters, etc. was by autoclaving at 121°C (1.05 Kg Cm^{-2} steam pressure) for 15 minutes.

(f) Selective media were supplemented as shown in Table 2-5. Supplements were sterilized by filtration through a Millipore HAWP membrane filter, 0.45 µm pore size (Millipore UK Ltd., Wembley, Middlesex).

The following media formulations were used:

(a) TY (Beringer, 1974)

	gl^{-1}
Difco Bacto-tryptone	5
Difco yeast extract	3
$CaCl_2 . 6H_2O$	1.3

(b) SY (Beringer, 1974)

	gl^{-1}
K_2HPO_4	0.22

The following were sterilized separately and added to the medium prior to use:

Addition	Stock solution gl^{-1}	ml added l^{-1}
$MgSO_4 . 7H_2O$	0.1	1
$CaCl_2 . 6H_2O$	0.04	1
$FeCl_3 . 6H_2O$	0.033	1

Table 2-5: Antibiotic supplements for media

Antibiotic (a)	Stock solution (mg ml^{-1}) (b)	Solvent	Concentration in media ($\mu g\ ml^{-1}$)	M.I.C. ($\mu g\ ml^{-1}$)(c)
Rifampicin	50	Dimethyl sulphoxide	50	0.5
Streptomycin	100	Distilled water	100	10
Kanamycin	100	Distilled water	100	50
Spectinomycin	100	Distilled water	100	20
Nalidixic acid	50	Distilled water	50	ND (d)
Tetracycline	5	Distilled water	2.5	0.05
Ampicillin	100	Dilute NaOH	50	5

(a) Rifampicin, kanamycin, ampicillin and tetracycline were obtained from Sigma Chemical Co. Ltd., London; streptomycin from Glaxo Laboratories Ltd., England; nalidixic acid from Calbiochem, San Diego, U.S.A.; spectinomycin from Upjohn Ltd., Crawley, Sussex.

(b) All stock solutions except tetracycline and ampicillin were stored in the dark at $4^{\circ}C$. Tetracycline and ampicillin were made up immediately prior to use.

(c) Minimum inhibitory concentration for R. trifolii P_3. This was determined as the lowest concentration of antibiotic causing complete inhibition of growth on an agar plate.

(d) ND - not determined.

Addition	Stock solution gl^{-1}	ml added l^{-1}
Na succinate	0.27	5
Na glutamate	0.22	5
Biotin Thiamine hydrochloride Calcium pantothenate	0.001	1

(c) Nutrient broth and agar

	gl^{-1}
Oxoid nutrient broth	25

(d) YEM (Vincent, 1970)

	gl^{-1}
K_2HPO_4	0.5
$MgSO_4.7H_2O$	0.2
NaCl	0.1
Mannitol	10.0
Difco yeast extract	0.4

(e) Seedling agar and nutrient solution (Jensen, 1942)

	gl^{-1}
$CaHPO_4$	1.0
K_2HPO_4	0.2
$MgSO_4.7H_2O$	0.2
NaCl	0.2
$FeCl_3$	0.1

Trace elements were then added as 1 ml of a stock solution

containing:

Bo	0.05%
Mn	0.05%
Zn	0.005%
Mo	0.005%
Cu	0.002%

Slopes were made by dispensing the agar medium into 150 x 19 mm tubes in 10 ml amounts.

Liquid medium was made at half the strength of the solidified medium.

BUFFERS

(a) Phosphate buffer, 0.067M, pH 7.0

	gl^{-1}
KH_2PO_4	4.56
Na_2HPO_4	4.75

(b) TES buffer, pH 8.0

	gl^{-1}
Tris	6.05
Na_2.EDTA	1.86
NaCl	2.92

CHEMICALS

Sodium lauryl sulphate (SLS), ethylene diamine tetra-acetic acid-disodium salt (Na_2.EDTA), hydroxymethyl methyl amine (Tris),

caesium chloride for ultracentrifuge work, and polyethylene glycol-6000 (PEG) were obtained from BDH Chemicals Ltd., England. Lysozyme (E.C.32.1.17., Grade 1; Sigma Chemical Co. Ltd.) was stored in a desiccator at 4°C.

MAINTENANCE OF CULTURES

Strains in routine use were subcultured monthly on TY agar plates from a single representative colony. Stock cultures, liquid suspensions of crosses and mutagenized strains were maintained in 20% glycerol at -20°C. Genetically marked strains and those carrying plasmids were routinely subcultured to supplemented media for characterization of the phenotype. Liquid cultures were grown in 5-10 ml amounts in 250 ml Erlenmeyer flasks on an orbital shaker incubator or a reciprocal shaking water bath. Rhizobium strains were incubated at 30°C Escherichia coli and Pseudomonas aeruginosa were incubated at 37°C.

Dilutions of cultures for counts of viable numbers were carried out in either TY broth or SY broth. These were 10-fold (0.5 ml into 4.5 ml) or 100-fold (0.1 ml into 9.9 ml).

ISOLATION OF GENETICALLY MARKED STRAINS

Antibiotic resistant strains were isolated by plating a suspension of the parental strain containing about 10^{10} v.u. ml^{-1}

(obtained by centrifuging a late-log phase culture and resuspending in one tenth the original volume) onto plates containing the appropriate antibiotic. Single colonies arising after 4 days incubation were restreaked for purification on the selective medium.

ISOLATION AND TESTING OF BACTERIA FROM NODULES

Nodules were sterilized by soaking in 95% ethanol (1 minute), followed by 0.1% mercuric chloride (3 minutes), then washing six times in sterile distilled water. Each individual nodule was crushed in 1 ml TY broth and dilutions were spread on TY plates and TY plates supplemented with appropriate antibiotics.

ISOLATION AND CHARACTERIZATION OF PLASMID DNA

The rationale for the isolation of plasmid deoxyribonucleic acid depends upon lysing host bacterial cells and subsequently treating the lysate so that the smaller circular plasmid DNA molecules are separated from the relatively huge mass of chromosomal DNA. An integral part of this requires either the sedimentation through an alkaline or neutral sucrose gradient or equilibrium density centrifugation with an intercalating dye such as ethidium bromide. The protocol employed in this study is that of Hansen and Olsen (1978). It includes some aspects of three extant lysis procedures (Guerry, Le Blanc and Falkow, 1973;

Humphreys, Willshaw and Anderson, 1975; Currier and Nester, 1976), and also incorporation of a heat pulse during lysis. Conditions of treatment for each step are summarized in Table 2-6.

Characterization by electron microscopy

To prepare plasmid DNA for electron microscopy, the crude plasmid preparation was centrifuged, after phenol extraction for the separation of proteins, at 36,000 rpm on an MSE Superspeed Ultracentrifuge in a caesium chloride ethidium bromide gradient. The plasmid band was collected from above using a syringe with a 19 gauge needle, extracted with cold CsCl saturated isopropanol to remove ethidium bromide and dialyzed against two changes of cold TES buffer. Analysis of DNA contour length using the basic protein film technique (Davis et al., 1971) was performed with a Silmens 101 electron microscope. Tracings of circular molecules of plasmid DNA were measured using a map measurer.

Plasmid molecular size was determined by the ratio of contour lengths using pSC101 as a 6.06×10^6 standard (Bukhari et al., 1977).

PREPARATION OF HIGH TITRE PHAGE LYSATES

A suspension of the phage was plated by the agar layer method (see below) to yield confluent plaques. This layer was then scraped off into 3 ml broth, thoroughly shaken and 0.2 ml chloroform added to kill any remaining bacteria. The suspension was shaken again,

Table 2-6: Isolation of plasmid DNA (a)

Step	Operation	Conditions
1	Cell growth	1 litre of cells in TY broth, to about 2×10^8 cells ml^{-1}.
2	Cell lysis	Washed cells resuspended at high osmolarity, cold; addition of lysozyme; Na_2EDTA, SDS to 4%; intermittent 55°C pulses.
3	Alkaline denaturation	pH 12.1-12.3, 3 minutes.
4	Neutralization	Addition of 2M Tris (pH 7.0) to lower pH to 8.5-9.0.
5	Removal of membrane-chromosome complexes	Addition of SDS to 4%, NaCl to 1M; refrigeration overnight; centrifugation 30 minutes at 12,000 rpm.
6	Concentration of plasmid DNA from supernatant	Addition of PEG 6000 to 10%; refrigeration for 6 hr; centrifugation at 2,500 rpm for 5 min; resuspension in about one fortieth volume.

(a) Hansen. J.B. and Olsen R.H., 1978.

centrifuged at 3,000 rpm on a bench centrifuge (MSE Angle Centrifuge) for 2-3 minutes. The supernatant was removed, passed through a Millipore membrane filter (pore size 0.45 μm, diameter 25 mm), and stored at 4°C. This method usually gave a phage lysate containing about 10^{10} plaque-forming units (p.f.u.) ml^{-1}.

DETERMINATION OF PHAGE TITRE

The phage titre was determined by the agar layer method. To tubes containing 3 ml of molten TY medium with half-strength agar (7.5 gl^{-1}), 0.1 ml amounts of an early log phase culture were added. Then 0.1 ml dilutions of the phage were added to the tubes which were gently rotated to mix the contents before pouring onto prepared plates of full strength TY agar. Plaques could be observed after incubation for 2 days at 30°C.

CONJUGATIONAL TRANSFER OF R-FACTORS

Transfer of R-factors was carried out using a membrane mating procedure (Jacob et al., 1976). Cultures of late log phase bacteria, about 10^{9} v.u.ml^{-1}, were used in crosses. In Rhizobium x Rhizobium crosses, 0.5 ml volumes of donor and recipient cultures were mixed in TY broth. In crosses involving other species 0.1 ml of donor culture was mixed with 0.5 ml of the recipient culture. The suspension was passed through a Millipore membrane filter (pore size 0.45 μm, diameter 25 mm), the bacteria

being retained on the filter which was then transferred to the surface of a TY plate. Following incubation, the bacteria adhering to the filter were scraped off, resuspended in 2 ml phosphate buffer, diluted and plated onto appropriate selective media.

MAPPING OF MUTANTS

The plasmid R68.45 mediates chromosomal transfer within and between several species of Rhizobium including R. trifolii (Beringer and Hopwood, 1976; Johnston and Beringer, 1977; Beringer, Hoggan and Johnston, 1978a). A kanamycin sensitive derivative, pJB_3JI, which has the same mode of transfer, was used for mapping purposes in this project. The first step was to construct a suitable donor strain from that carrying the genes to be mapped, by transfer of the plasmid. A series of crosses were then carried out with nine recipient strains of R. leguminosarum, each carrying genetic markers two of which flanked a section of the bacterial chromosome, the nine sections making up the whole chromosome (see Fig. 2-1 and Table 2-2). When Rhizobium recipients receive a section of the chromosome flanked by two genetic markers, any marker located between these will nearly always be inherited with them (Beringer et al., 1978a). It was suggested that this effect might be the result of poor recombinational ability, at least when this plasmid was used to promote chromosome transfer. Alternatively, there might be some sort of

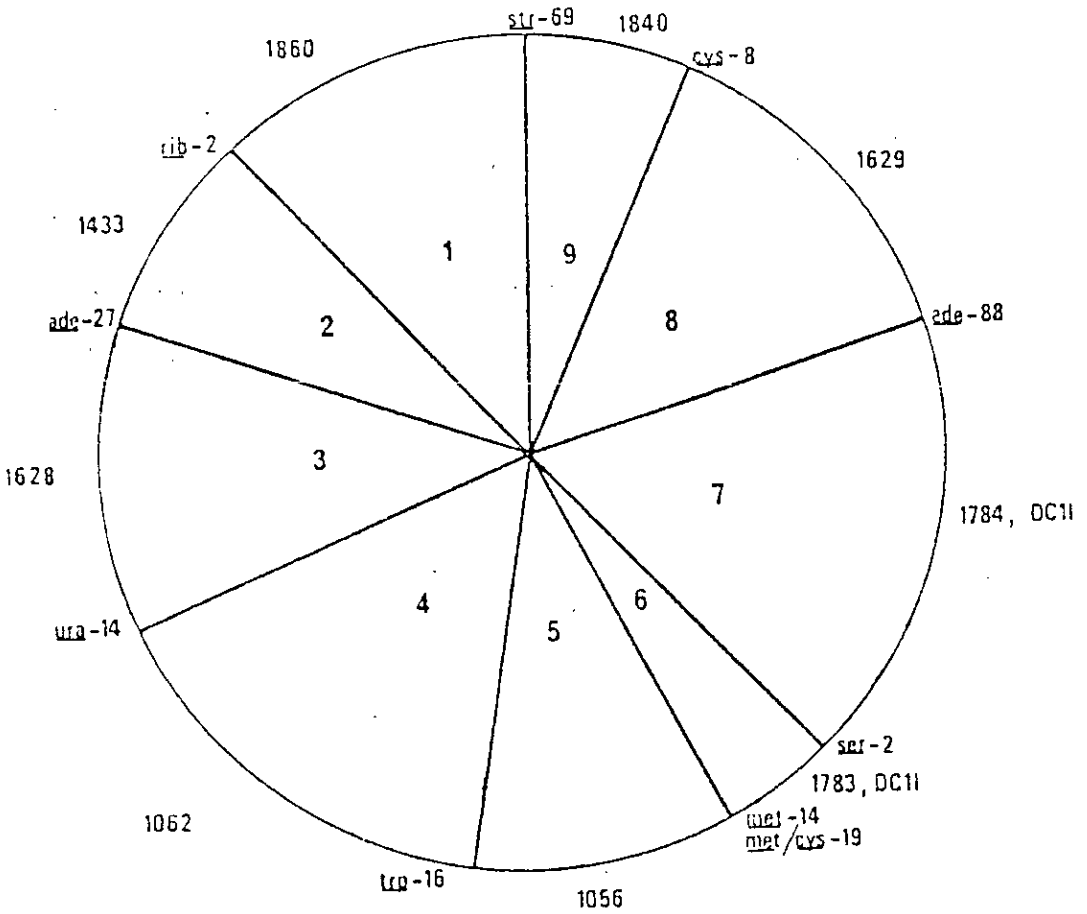


Fig. 2-1: Map of the chromosome of Rhizobium leguminosarum showing sections used for mapping. The strains carrying the flanking markers for each section are indicated (J.E. Beringer, personal communication).

"end effect" due to preferential recombination at the ends of incoming fragments.

It was therefore possible to select for the transfer of two donor alleles, e.g. ura$^+$ and ade$^+$ in a cross with R. leguminosarum 1628 as recipient and then to test the recipient bacteria for co-inheritance of the genetic marker to be mapped. The marker was then assigned to the section. A more accurate position for the marker within the section could be obtained from an analysis of co-inheritance percentages with the flanking markers. The co-inheritance percentages are the frequencies with which recipients selected for one chromosomal marker also receive a second non-selected marker. Details of the crosses are given in the relevant chapter.

GROWTH OF CLOVER PLANTS

The plants were grown in a greenhouse with a minimum temperature of 20°C, unless otherwise indicated, and a 16 hour daylength. They were illuminated by banks of two warm-white and two daylight fluorescent tubes (Crompton) positioned 71 cm above bench level and supplying 29,000 lux at plant height.

PREPARATION AND INOCULATION OF STERILE CLOVER PLANTS

The seeds were sterilized by treating them for 15 minutes with concentrated sulphuric acid. The sterilized seeds were transferred to plates of yeast-mannitol agar since TY medium gave only low germination percentages. The plates were inverted and incubated in the dark for 2 days. Contaminated and apparently abnormal seedlings were then discarded.

The seedlings were transferred to prepared growth tubes and placed in the greenhouse for 2 days, to allow them to establish. The growth tubes were held in wooden blocks 8.7 cm x 37.5 cm x 4.0 cm into which holes 2 cm diameter by 3.2 cm depth had been drilled. Thus the roots obtained some degree of shading. The growth tubes were spaced 2 cm apart within the rack. Following 2 days growth the plants were inoculated by adding a suspension of the bacteria (about 10^7 v.u.ml^{-1}) in liquid Jensen's medium, sufficient to cover the roots of the seedling. Plants were then grown for 5 to 6 weeks, liquid growth medium being added as required. All experiments included both uninoculated controls and nitrogen-supplied controls which contained 10mM NH_4NO_3 in both liquid and solid medium. Nodules began to appear approximately 10 days after inoculation, and differences between treatments, e.g. inoculated and uninoculated, generally became apparent 3 to 4 weeks after inoculation.

PREPARATION AND SAMPLING FOR THE ACETYLENE REDUCTION ASSAY

For acetylene reduction assays the cotton wool stopper of the test tubes was replaced by a rubber Subaseal (Gallenkamp & Co. Ltd.) and 2 ml of acetylene (British Oxygen Corporation Ltd.) injected into the tube. The tubes were kept in the greenhouse for 10 hours before sampling, unless otherwise indicated. Samples of 0.2 ml for assay were taken up into 1 ml plastic disposable syringes (Becton, Dickinson & Co. Ltd.). Where many samples were taken at one time, the syringes were stuck into rubber bungs until they could be assayed. No appreciable loss of gas was observed over a period of several hours. A useful review on the preparation of material for the acetylene reduction assay, and details of the assay procedure, is given by Masterson & Murphy (1980).

THE ACETYLENE REDUCTION ASSAY

Gas liquid chromatography of samples was carried out on a Pye 104 gas-liquid chromatograph fitted with a 152 cm column packed with Porapak R (PhaseSep Ltd., Deeside, Clwyd). The nitrogen carrier gas had a flow rate of 20 ml min^{-1}; the hydrogen pressure was 1.05 kg cm^{-2}; and the air pressure 1.5 kg cm^{-2}. The column oven was held at 50°C and the detector oven at 150°C. Samples of 0.2 ml were injected into the column and ethylene and acetylene gases identified by the position of their peaks on the chart

recording, the peak of ethylene emerging immediately before the peak of acetylene. Ethylene was measured by a comparison of the peak height produced with that of a 0.2 ml standard sample containing 9.69 nmoles of ethylene in that volume (PhaseSep Ltd.).

CHAPTER III

"EXPERIMENTAL INVESTIGATIONS"

PART I

"INVOLVEMENT OF PLASMIDS IN SYMBIOSIS"

Screening for plasmids in strains of R. trifolii and estimation of their molecular weights:

The isolation procedure for large plasmid DNA molecules, summarized in Table 2-6, was used with five symbiotically-effective strains of R. trifolii: TA-1, FA-6, P_3, 204 and 1-DL. After ultracentrifugation of their cell lysates in CsCl-EtBr density gradients, two fluorescent bands under UV irradiation were clearly seen for strains TA-1, FA-6, P_3 and 204. Only one band was detected for strain 1-DL in the position of the linear DNA. The failure to detect supercoiled DNA in strain 1-DL may have been due to limitations of the method used or it may reflect an actual absence of extrachromosomal DNA. DNA taken from plasmid bands of strains TA-1, FA-6, P_3 and 204 was used directly for electron microscopy. Micrographs were taken at 20,000x magnification (see Fig. 3-1) and these were magnified 5x in a De Vere 54 Varicon projector for tracing. By measuring the contour length of the molecules examined and that of pSC101 as a standard, it was possible to estimate the molecular weight of the plasmids. The range of the sizes was from 7 ± 0.2 to $220 \pm 0.2 \times 10^6$ (Table 3-1). This range of molecular weights is in general agreement with previously reported values for plasmids in Rhizobium (see Chapter I). However, it is worthy of note that plasmids of molecular weight less than 20×10^6 are rarely found in Rhizobium and these may well be suitable as cloning vectors for genetic engineering. Three strains

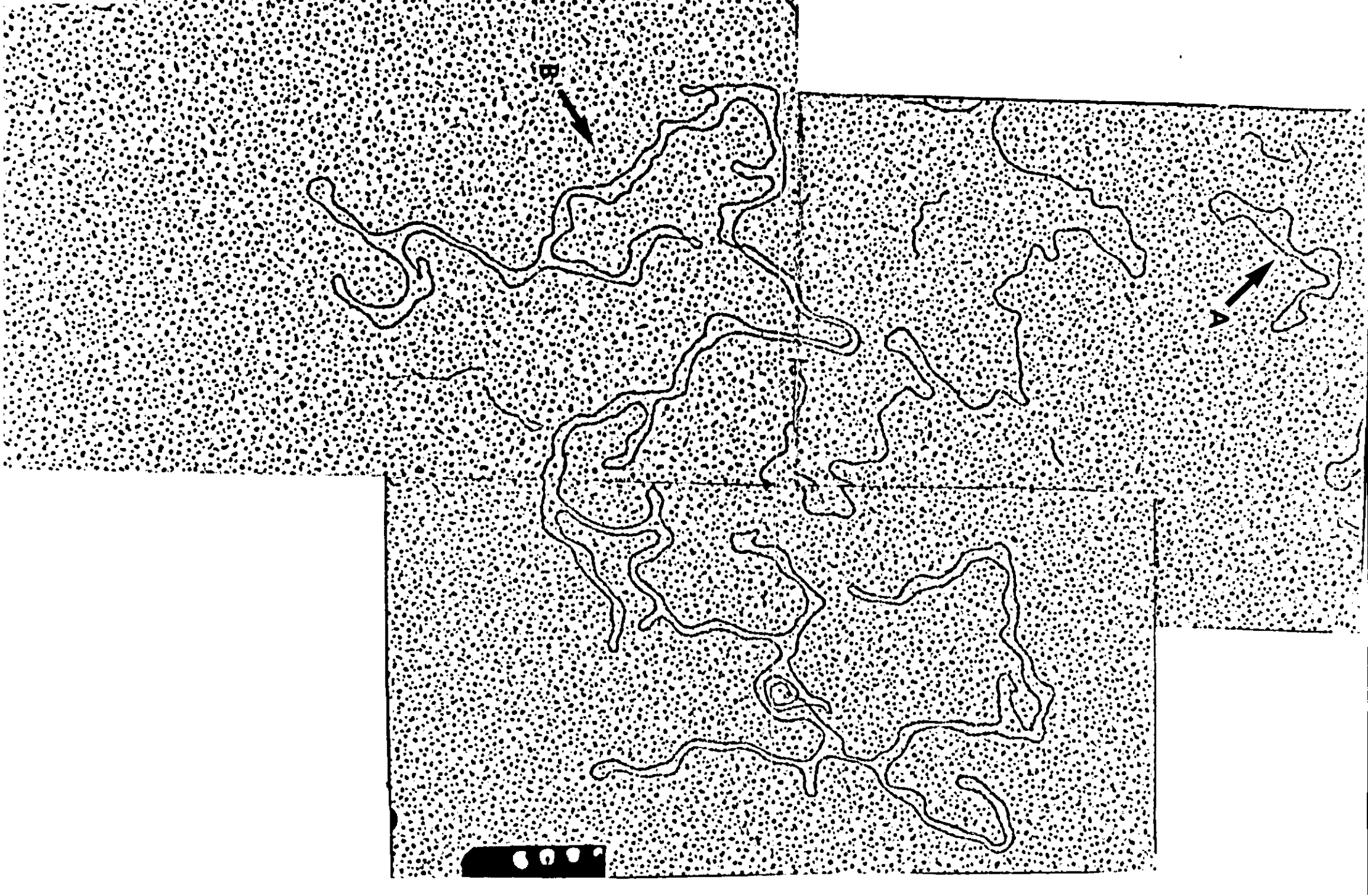

B
A

Table 3-1: The electron-microscopic determination of molecular weights of the plasmids isolated from different strains of R. trifolii

Strain	Plasmid	No. of molecules measured	Molecular weight (x 10^6)
FA-6	pMAM-1a	12	26 ± 0.3
	pMAM-1b	7	62 ± 0.4
	pMAM-1c	6	82 ± 0.6
	pMAM-1d	4	127 ± 0.5
TA-1	pMAM-2a	12	7 ± 0.2
	pMAM-2b	11	30 ± 0.2
204	pMAM-3a	10	18 ± 0.2
	pMAM-3b	7	65 ± 0.4
P_3	pMAM-4	4	220 ± 0.2

of R. trifolii: FA-6, TA-1 and 204 contain more than one size-class of plasmid. In the preparations examined using electron microscopy, the small plasmids (MW $< 30 \times 10^6$) occurred two to three times more frequently than the larger plasmids. This may represent their actual frequency in the cell, or the small molecules may have arisen through the occasional breakage of large plasmids, a phenomenon observed for some R-factors (Nisioka, Michiko and Clowes, 1969). However, although the strains showed an apparent diversity in their plasmid spectrum, they had indistinguishable sensitivity profiles to six antibiotics (Table 2-5) with no significant resistance to any individual antibiotic. This excludes the possibility of any plasmid detected being an R-factor. Of particular significance was the detection of a single-size class of plasmid in strain P_3. The estimated molecular weight was $220 \pm 0.2 \times 10^6$. The concentration of the purified plasmid DNA was measured spectrophotometrically, the calculation being based on the assumption that an absorbance of 1.0 at 260 nm corresponds to 50 μg DNA ml^{-1}. Between 200 and 600 μg of plasmid DNA was obtained in different experiments from three gm wet weight of bacteria. These yields are compatible with those expected for cells containing one copy of plasmid per genome. The plasmid isolated from strain P_3, designated pMAM-4, was chosen as a candidate on which to study the hypothesis of plasmid-participation in symbiosis. The reasons for this choice were that

pMAM-4 is present in P_3 as a single-size class, which excludes the possibility of the dispersion of plasmid-borne symbiotic genes on different plasmids, and because of previous genetic studies on its host (Cunningham, 1979).

However, to build up any form of genetic evidence for such participation, it is necessary to have a genetic marker on the plasmid under study in order to assess its presence or absence in a cell.

In vivo genetic labelling of plasmid pMAM-4:

For biologically labelling the plasmid pMAM-4, the vector pJB4JI was employed (Beringer et al., 1978b; see chapter I). Briefly, the method relies upon the fact that P1-incompatibility-group plasmids into which the bacteriophage Mu genome has been inserted can be transferred into Rhizobium but fail to become established. To make pJB4JI, a Mu-containing derivative of the gentamicin-resistant P-group plasmid, pPH1JI, was constructed in E. coli, and into this Tn-5 was inserted. An E. coli strain (1830) carrying this hybrid plasmid, was crossed to R. trifolii strain (MD-1b), a rifampicin-resistant derivative of strain P_3, on a filter membrane (see chapter II for method). After incubation at $30^{o}C$ for 24 hrs, the mating mixture was spread on TY agar plates supplemented with rifampicin (50 $\mu g\ ml^{-1}$) to counterselect the E. coli donor, and kanamycin (100 $\mu g\ ml^{-1}$) to select for Tn-5

transfer. Such selection picks out those bacteria in which Tn-5 has left the "suicide" plasmid and inserted into either the chromosome or the resident plasmid in R. trifolii. As a control, R. trifolii (MD-1b) was plated on TY Kan; spontaneous mutation to kanamycin resistance occurred at a frequency of less than 10^{-8}. Transconjugants resistant to 100 μg ml^{-1} of kanamycin were selected; they arose at a frequency of 5×10^{-6} per recipient.

To pick up those clones in which Tn-5 had inserted into the resident plasmid, transconjugants were tested in patch crosses for the ability to transfer Kan at high frequency to R. leguminosarum strain (1056). For patch crosses, master plates were replica plated, using sterile velvet onto the surface of selective TY plates each spread evenly with 0.1 ml of an exponentially-growing culture of the recipient. The selective plates were supplemented with streptomycin (100 μg ml^{-1}) and kanamycin (100 μg ml^{-1}). The plates were then incubated for 24-36 hrs at 30°C. Confluent growth of clearly distinguishable recombinant colonies were observed. Out of 250 presumptive recombinants, 220 carried the expected auxotrophic and antibiotic-resistance markers of the strains from which they were derived, 20 of which were chosen for further study.

Kanamycin-resistant derivatives of strain (MD-1b), designated (MD-2b, a to t), that gave rise to the 20 recombinants were tested subsequently in filter mating (see chapter II for method) with

R. leguminosarum (1056) to assess the actual frequency of Kan transfer. Transconjugants at a mean frequency of 4×10^{-6} were obtained. The self-transmission of the resident plasmid pMAM-4 can be deduced from this result, as the frequency is too high to be explained by mutation of the parent cells to antibiotic resistance. However, the low frequency may be attributed to entry exclusion/ incompatibility with the resident plasmids in strain (1056), the parent of which was shown to carry at least four plasmids of MW90 to 200×10^{6} (Hirsch et al., 1980). These results demonstrated the conjugative ability of plasmid pMAM-4.

No further work was carried out on the plasmid location of Tn-5 insertion, which can be ascertained by DNA-DNA hybridization (Southern, 1975), because it became clear from the results of Nuti et al. (1979), Stanley and Dunican (1979) and Zurkowski and Lorkiewicz (1979), during the course of this work, that plasmids in Rhizobium could carry genes involved in symbiosis.

Symbiotic phenotypes of the interspecific recombinants:

R. leguminosarum recombinants, designated (MD-1d, a to t), that inherited the auxotrophic and antibiotic-resistance markers, were selected randomly from crosses involving all 20 R. trifolii (MD-2b, a to t) donors. These were used to inoculate clover. Individual colonies were transferred to 1 ml TY broth in test tubes and incubated for 2 days at $30^{\circ}C$. The cultures were diluted 100-fold into Jensen's liquid medium and each used as an inoculum for

10 replicates of clover plants (for plant growth conditions, see chapter II). Strain (MD-2ba) and strain (1056) were included as controls. Nine recombinants formed ineffective nodules (assayed by acetylene reduction, see chapter II for method). However, nodulation was delayed by about one week and the number and size of nodules were reduced compared with those in the (MD-2ba) control. Strain (1056) did not nodulate clover. Nodulation by the recombinants mentioned above showed that some "nodulation" genes are located on the plasmid pMAM-4 or are co-transferred at comparatively low frequency with this plasmid. However, in 11 cases "clover nodulation" was not co-transferred with Kan. These cases could be explained by any of the following possibilities:

1. The insertion of Tn-5 into a transmissible nodulation gene.
2. The insertion of Tn-5 into another cryptic plasmid that does not carry nor co-transfer nodulation genes.
3. The inability of nodulation genes to be transferred and become established intact in the recipients.

Sensitivity of R. trifolii carrying the plasmid pMAM-4 to the donor-specific phages PRR1 and PR4:

The phage PRR1 adsorbs specifically to the sides of pili coded by Inc P1 plasmids (Bradley, 1976) and therefore lyses only bacteria carrying P1-plasmids. A group of other donor-specific phages, of which PR4 (Bradley, 1976) is an example, attaches to the tips of

both Inc P1-determined pili and morphologically different pili determined by plasmids of Inc W and Inc N groups (Bradley and Cohen, 1976). The sensitivity of strain P_3 to PRR1 and PR4 (10^8 p.f.u. ml^{-1}) was tested by using the agar layer method (see chapter II), using (MD-1e), P. aeruginosa strain (PAO) carrying RP4, as a control. However, it was not possible to demonstrate the multiplication of PRR1 and PR4 in strain P_3 as no plaques were observed after three days of incubation at 28°C, whereas strain (MD-1e) carrying RP4 acted as an efficient host for the multiplication of these phages leading to the formation of plaques. A similar observation was made by Hirsch (1978) when assessing sensitivity of R. leguminosarum pPH1JI to the donor-specific phages. She explained this inhibition of plaque formation as the result of "the masking effect of exopolysaccharides slime produced by R. leguminosarum". However, two other possibilities exist:

1. The reduction in the efficiency of plating may be due to lower conjugal transferability observed with plasmid pMAM-4, since both efficient plasmid transfer and phage susceptibility are under the same regulatory control, a phenomenon known to be associated with some R-factors (Stanisich, 1974).
2. Host-controlled restriction and modification may be exhibited by strain P_3.

The latter possibility was examined by performing an experiment illustrated in Fig. 3-2. The host ranges of PRR1 and PR4 were

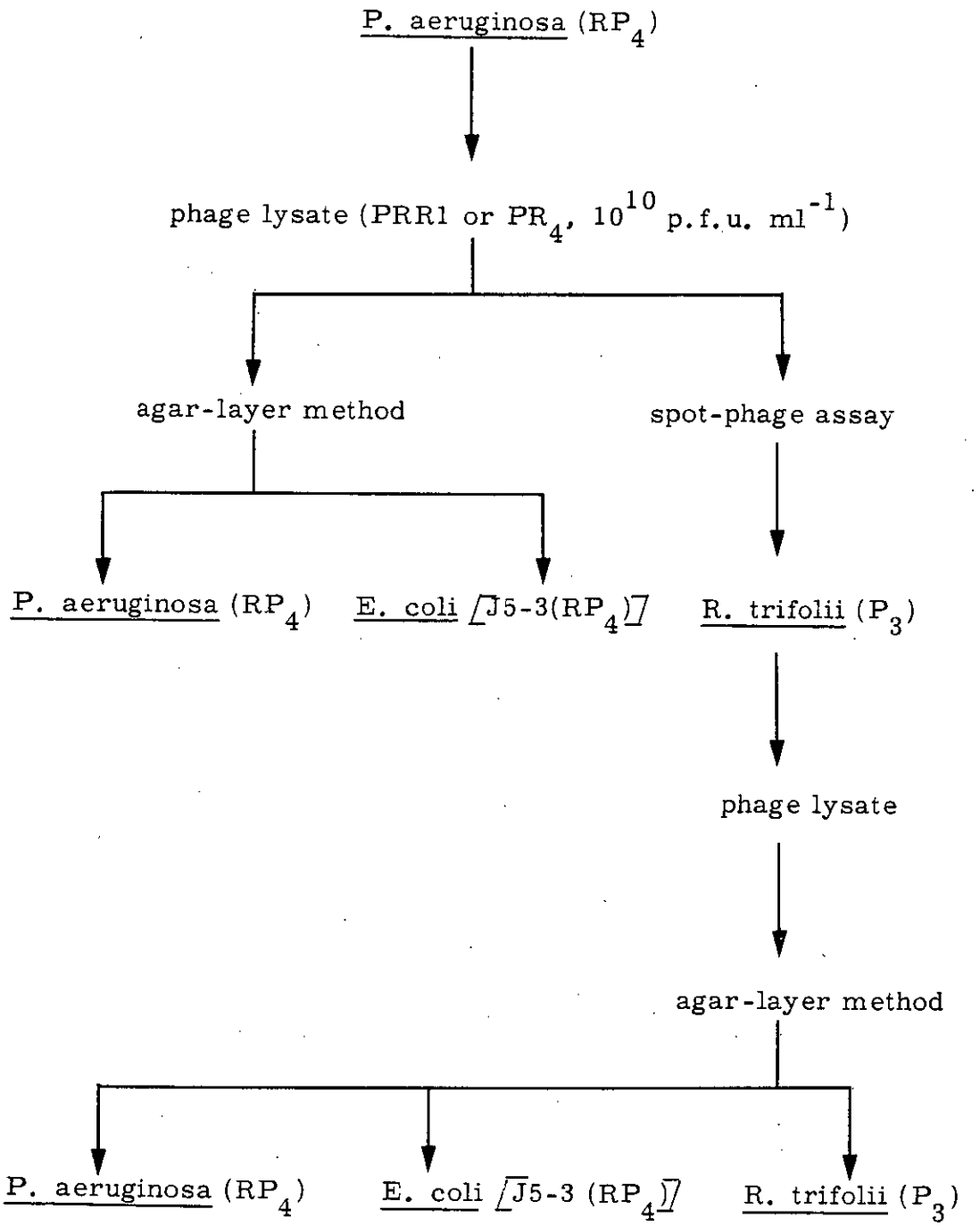


Fig. 3-2: A diagram showing the protocol used in demonstrating restriction and modification in R. trifolii (P_3).

determined by using the agar layer method with (MD-1e) and E. coli [J5-3 (RP4)], the latter being included as an additional control and by using the spot phage assay with P_3. In the latter assay, bacteria were seeded on the surface of TY medium by flooding the agar surface with 2 ml of a log-phase culture and removing the excess fluid. When the surface was dry, 50 µl of 10^{10} p.f.u. ml^{-1} phage lysate (see chapter II for method) was spotted onto the bacterial lawn using sterile micropipettes. For every tested culture, one plate seeded with bacteria was prepared without adding the phage lysate.

No restriction was observed of the phages grown on P. aeruginosa and tested on E. coli. A positive response of strain P_3 was observed in the spot phage method as a partial clearing in the lawn. It was assumed that this clear area contained those phage molecules that had escaped restriction and survived. The cleared areas were used to prepare phage lysates, the titres of which were determined by the agar layer method on P_3,(MD-1e), and [J5-3 (RP4)]. The P_3.PR4 lysate was shown to be of $\leq 10^6$ p.f.u. ml^{-1} on P_3,(MD-1e) and [J5-3 (RP4)]. The P_3.PRR1 lysate contained $\leq 10^3$ p.f.u. ml^{-1} on the above-mentioned hosts. The efficiency of plating values was based on averages of three independent experiments with each member. The p.f.u. ml^{-1} is indicated as less than a certain value because of abnormal plaque morphology which made the plaque count subject to error. From

these values, the reduction in the efficiency of plating of phages PR4 and PRR1 on P_3 is due to restriction of most of the phage DNA molecules.

Since the inability of R. trifolii strain P_3 to propagate the donor-specific phages grown on P. aeruginosa was due to restriction and that surviving phage DNA molecules plaqued at the same titre on P. aeruginosa, E. coli and R. trifolii (P_3), it can be concluded that the plasmid pMAM-4 belongs to the incompatibility group P1.

PART II

"ISOLATION AND MAPPING OF SYMBIOTIC-DEFECTIVE MUTANTS"

The isolation of symbiotic-defective mutants was achieved using two methods: firstly, growing bacteria at an elevated temperature, in the hope that some of the cells which survived were cured of their plasmid DNA and had altered symbiotic properties. Secondly, transposon-mutagenesis and assessment of the symbiotic properties of the clones isolated.

1. Attempt to cure R. trifolii of its resident plasmid:

An attempt was made to isolate plasmid-free mutants which could be used as recipients to study the expression of foreign DNA in Rhizobium. The protocol was to incubate bacterial cultures at elevated temperatures (Zurkowski and Lorkiewicz, 1978), isolated clones of which could then be assessed for their symbiotic properties. The strains of R. trifolii chosen originally for this study were (MD-1b) and (MD-1a). The optimal temperature for the growth of both of these strains was 28°C. However, the maximum temperature for the growth of strain (MD-1b) was 32°C, while strain (MD-1a) showed some growth even at 37°C. Strain (MD-1a) was selected therefore for this experiment. A culture was incubated at 37°C for 48 hrs, and the bacteria plated on TY agar medium to give single colonies. These were transferred to agar slants and the symbiotic properties of the clones determined in test tubes by inoculation of sterile clover seedlings with samples of the isolated clones. The experiment was terminated after five weeks. Putative

Nod^- and Fix^- mutants were re-tested using five replicates for each clone. Out of 100 clones tested, nine showed the Nod^- phenotype. The Nod^- mutants, designated (MD-2a to 10a), remained non-nodulating on re-testing; reversion to the Nod^+ phenotype was not observed. The provenance of the isolated Nod^- mutants was confirmed to exclude the possibility that they were contaminants. All Nod^- mutants showed the same level of resistance to spectinomycin as the parental strain, as well as showing the same sensitivity to the rhizobiophage Rt1 (Cunningham, 1979). In addition to the Nod^- mutants, another five clones were isolated that nodulated clover but were completely ineffective. These Fix^- mutants were designated (MD-11a to 15a). Dry weights of plants nodulated by (MD-11a to 15a) were significantly lower than those nodulated by the parental strain (MD-1a) (see Table 3-2). It was demonstrated that strain 204, the parental strain of (MD-1a), carried at least two plasmids (see Part I). The Nod^- and Fix^- phenotypes obtained after heat treatment were probably the result of loss of the resident plasmid(s) or possibly due to a deletion in the plasmid(s).

2. Tn-5 mutagenesis:

A cross between *E. coli* (1830) and *R. trifolii* (MD-1b) was set up. Following incubation, the bacteria from this cross were resuspended in TY broth and plated onto an appropriate selective

Table 3-2: Dry weights of clover plants uninoculated or inoculated with parental or Fix$^-$ mutants of R. trifolii

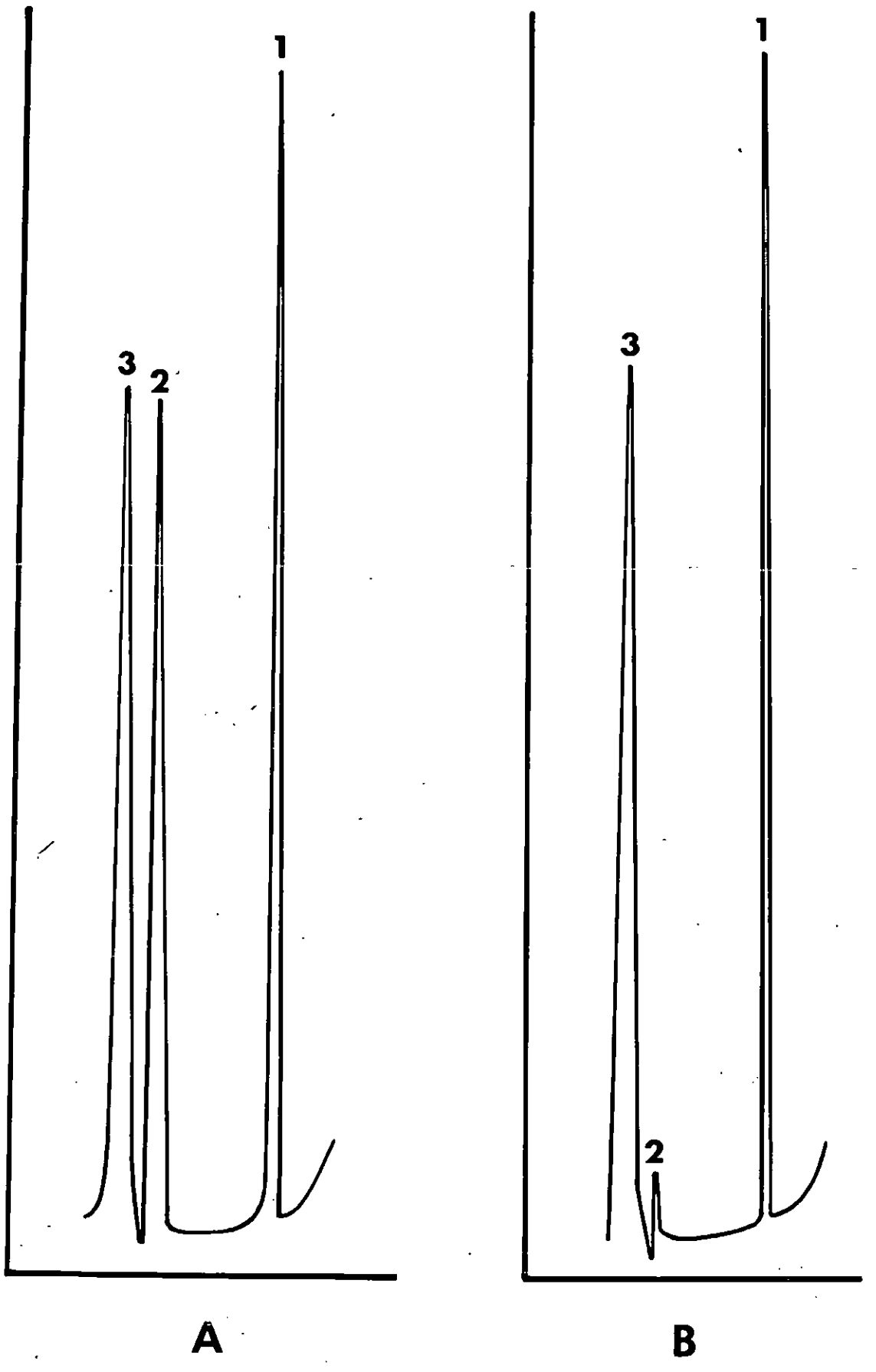
Inoculum strain	Dry weight (mg) (a)
MD-1a	96.8 $\pm$ 28
MD-11a	22.3 $\pm$ 9.5
MD-12a	24.0 $\pm$ 9.6
MD-13a	20.3 $\pm$ 8.8
MD-14a	19.0 $\pm$ 8.6
MD-15a	22.5 $\pm$ 9.9
None: uninoculated plants	13.2 $\pm$ 8.4

(a) Mean value of 5 replicates $\pm$ 1 standard deviation.

medium to detect the transfer of Tn-5 to R. trifolii. The frequency of transfer was 10^{-6} and a total of 1,000 colonies was obtained following incubation. Individual colonies were transferred to 1 ml TY broth in test tubes and incubated for 2 days at 30°C. The cultures were diluted 100-fold in Jensen's medium and each was used as an inoculum for an individual clover plant. A 0.5 ml volume from each culture was also diluted, with an equal volume of 40% glycerol, and stored at -20°C. The plants were grown for 5 weeks and acetylene reduction assays were carried out as described in chapter II.

Putative non-fixing mutants were retested, five replicates being used for each clone. Plants were grown at both 15°C and 25°C. Those at 15°C ($\pm$ 1°C) were illuminated by Wotam mercury iodide lights delivering 63,000 lux at plant height. Plants at 25°C ($\pm$ 2°C) were grown in a greenhouse with lighting as described previously (see chapter II). Out of 1,000 clones screened, a total of five Fix^- mutants were isolated. The mutants, designated (MD-3b to 7b), had considerably decreased acetylene reducing ability, i.e. nitrogenase activity, relative to the parental strain at both 15°C and 25°C (see Fig. 3-3). All of the mutants were defective in acetylene reducing ability at both temperatures suggesting that they were not temperature sensitive in nitrogen fixation. None of the mutants isolated was auxotrophic and their growth rates were not greatly different from that of the parental

Fig. 3-3: Separation of hydrocarbons by gas chromatography. Peaks: (1) methane; (2) ethylene; (3) acetylene. (A) strain (MD-1b); (B) (MD-3b) at 15°C.


1
3
2
A
1
3
2
B

strain, in so far as they produced similar sized colonies on TY agar after 3 days incubation at 30°C. All the mutants nodulated clover plants at the same time as the parent (MD-1b).

Mapping of the sites of transposon insertion that give rise to the Fix$^-$ phenotype:

Appropriate donor strains were constructed by transferring the plasmid pJB3JI from E. coli (1843) to R. trifolii (MD-3b to 7b). Selection was made for tetracycline resistance. Tetracycline resistant transconjugants arose at a frequency of 10^{-2} per recipient bacterium. One clone from each cross was purified and used in crosses with appropriate recipient strains of R. leguminosarum (see Table 2-2). Screening was for the transfer of kanamycin resistance and the appropriate prototrophic markers. The results of these crosses are shown in Table 3-3, from which it is clear that all strains were Cma$^+$ but with differing levels of efficiency. This variability could have resulted from strain differences or from the fact that the chromosome mobilizing ability of the parental plasmid R68.45 is unstable (Holloway, 1979). However, each mutant mapped showed two to three possible sites for Tn-5 insertion. This meant that further analysis, to map the accurate position of the insertion responsible for the symbiotic defective phenotype, was difficult. Models of transposon insertion which may explain this observation will be discussed later.

Table 3-3: Mapping of Tn-5 insertions

R. trifolii donor	*R. leguminosarum* recipient	Map section	Frequency of inheritance of selected markers (per recipient)
MD-8b	1062	4	1.3×10^{-7}
	DC-11	6	2.2×10^{-6}
MD-9b	1433	2	5.0×10^{-7}
	1056	5	2.2×10^{-6}
	1062	4	4.1×10^{-6}
MD-10b	DC-21	7	7.5×10^{-7}
	DC-11	6	3.1×10^{-6}
MD-11b	1062	4	5.0×10^{-7}
	1433	2	3.4×10^{-6}
MD-12b	1056	5	1.1×10^{-7}
	1629	8	9.5×10^{-7}

PART III

"A STUDY ON THE COMPETITIVE ABILITY OF THREE STRAINS OF RHIZOBIUM TRIFOLII"

Effect of temperature on competition amongst strains of Rhizobium trifolii:

The original Rhizobium strains used in the present investigation were selected after a preliminary study of nodulation and nitrogen fixation. Strain P_3 and 1-DL were from the United Kingdom and strain 204 from Iraq. The reason for comparing two U. K. strains with an Iraqi strain is the expectation that the Iraqi strain would perform better in nodulation than the U. K. ones as the temperature increased, since it showed high tolerance to temperature under laboratory conditions (see Part II). However, all nodulated Trifolium repens effectively at 20°C. Mutants resistant to antibiotics were isolated as described in chapter II. These were as follows: strain (MD-1a), a spectinomycin-resistant derivative of strain 204; strain (MD-1b), a rifampicin-resistant derivative of strain P_3 and (MD-1c), a streptomycin-resistant derivative of strain 1-DL. Inocula consisted of an intended 1:1 mixture in the following combinations:

A - (MD-1b) + (MD-1c) - tested at 20°C

B - (MD-1b) + (MD-1a) - tested at 15°C and 25°C

C - (MD-1c) + (MD-1a) - tested at 15°C and 25°C

The 1:1 mixture of strains was prepared by mixing equal proportions (0.5 ml) of the two suspensions of bacteria, the concentrations of which were adjusted to 5×10^7 bacteria ml^{-1}. This concentration was chosen in an attempt to exclude the spontaneous acquisition of

resistance to the antibiotics used as selective agents, i.e. the occurrence of nodules containing more than one strain of R. trifolii would not be due to a clone derived from a spontaneous mutant arising during the growth of the bacteria in the course of nodule development.

The plants (for growth conditions see chapter II and Part II, chapter III) were inoculated with samples of appropriate bacterial suspensions. A number of well separated and suitably sized nodules were isolated from clover plants about 5 weeks after inoculation. However, for several combinations this was not possible because large numbers of nodules were closely grouped together and single nodules could not be isolated. The number of nodules yielding data was further reduced because no bacteria could be isolated from many of the smaller nodules. Either bacteria were not present or were killed by the sterilization procedure. Thus the data are biased in favour of isolates from larger nodules. The presence of rifampicin-resistant, streptomycin-resistant or spectinomycin-resistant populations was detected by plating the macerates on TY medium supplemented with the appropriate antibiotic. Tabulated results for nodule strain identity are given in the Appendix.

In the combination (MD-1b) plus (MD-1c), the two strains competed equally for nodulation at 20^{o}C, as they form equal numbers of singly infected nodules. However, the competitive ability for

nodulation of these two strains was very evident when they were combined separately with (MD-1a). At 15°C, out of 101 nodules examined from the inoculation combination of (MD-1c) and (MD-1a), none contained (MD-1a) alone. When (MD-1b) and (MD-1a) formed the mixed inoculum only one nodule out of 84 contained (MD-1a) alone. However, strain (MD-1a) performed better at 25°C as the incidence of single infection by this strain increased to 6.3% when combined with (MD-1c) and to 13% when combined with (MD-1b). Double strain occupancy showed similar variation at different temperatures. In the combination (MD-1b) plus (MD-1a), the percentage of double strain occupancy increased from 7.1% at 15°C to 17.8% at 25°C. The combination (MD-1c) plus (MD-1a) showed a similar increase, i.e. from 6.9% at 15°C to 15.9% at 25°C. However, the percentage of double strain occupancy was 33.3% in the combination (MD-1b) plus (MD-1c). This high percentage may be attributed to their equality in competition for nodule formation. In the mixedly-infected nodules from all these inoculant mixtures, the observed populations of strains did however deviate from the 1:1 ratio of the inoculum (see Table 3-4). Strain (MD-1c) formed the majority of the population in all combinations. At 15°C, the average population in nodules mixedly infected with (MD-1c) and (MD-1a) was made up of 1.1×10^4 cells ml^{-1} of (MD-1c) and 1.8×10^2 of (MD-1a). At 25°C, the average population of (MD-1c) was 1.4×10^4, while that of (MD-1a) was 5.9×10^3. In the combination

Table 3-4: Average numbers of strains present in mixedly infected nodules at different temperatures

Mixture	Temperature		
	15°C	20°C	25°C
(MD-1b))		1.5×10^2	
)			
(MD-1c))		1.5×10^4	
(MD-1b))	2.0×10^3		7.5×10^3
)			
(MD-1a))	5.8×10^2		5.7×10^3
(MD-1c))	1.1×10^4		1.4×10^4
)			
(MD-1a))	1.8×10^2		5.9×10^3

(MD-1b) plus (MD-1c), the average population of strain MD-1b was 1.5×10^2, while the average population of strain MD-1c was 1.5×10^4. However, when strain (MD-1a) and (MD-1b) were present in the same nodule their numbers, at 25°C, were approximately equal, viz. 5.7×10^3 and 7.5×10^3 respectively. At 15°C, some deviation was observed, the average population of strain (MD-1b) being 2×10^3 cell ml^{-1}, while that of (MD-1a) was 5.8×10^2.

CHAPTER IV

"DISCUSSION"

1. PLASMIDS IN RHIZOBIUM TRIFOLII

(a) Screening for plasmids in R. trifolii

Attempts to isolate and positively identify plasmids in Rhizobium, particularly the large plasmids, were largely unconvincing or negative until improved cell lysis and DNA isolation procedures were used. The "gentle lysis" methods used generally for bacteria such as E. coli were unsatisfactory for rhizobia (Schwinghamer, 1981). However, following "complete lysis" involving the use of lysozyme, ionic detergents, proteases and other agents, a variety of procedures have been employed to look for large plasmids in different Rhizobium strains, i.e. alkaline sucrose gradients followed by the measurement of DNA renaturation kinetics (Nuti et al., 1977; Prakash et al., 1980), ultracentrifugation in CsCl-EtBr gradients followed by electron microscopy (Casse et al., 1979) and agarose gel electrophoresis (Hirsch et al., 1980). Factors pertinent to the isolation of large plasmids were discussed by Hansen and Olsen (1978). On the basis of their considerations, they devised a novel protocol incorporating aspects of three, then extant, procedures (Guerry, Le Blanc and Falkow, 1973; Humphreys, Willshaw and Anderson, 1975; Currier and Nester, 1976) to achieve reproducible isolation of large plasmids. Strict lysis conditions were defined that appeared to improve the separation of plasmid from chromosomal

DNA. The results in this thesis have confirmed the usefulness of this protocol. It was simple and reproducible, allowing the isolation of plasmids of molecular weight greater than 100×10^6. However, large plasmids seem to be a general feature of Rhizobium since they have been found to be abundant in different Rhizobium strains (Badenoch Jones et al., 1981; Dénarié et al., 1981; Dunican et al., 1981). An answer to the significance of the large plasmids in symbiosis may come from estimating the amount of extrachromosomal genetic information, i.e. from 3 to more than 10%, assuming that the Rhizobium chromosome is of a similar size to that of E. coli. It is now obvious that future Rhizobium breeding for legume inoculation will require not only chromosomal recombination (Kondorosi et al., 1977) but also plasmid transfer and recombination (De Jong, Brewin and Phillips, 1981).

(b) Genetic functions located on an indigenous plasmid in R. trifolii:

The plasmid pMAM-4, which originated in R. trifolii strain P_3, was chosen to investigate the role of plasmids in symbiosis because of its presence as a single-size-class in P_3 (see chapter III) and of previous genetic studies on its host (Cunningham, 1979). Using a technique for transposon-mutagenesis (Beringer et al., 1978b) a derivative of pMAM-4 was obtained which contained the transposon Tn-5 conferring resistance to kanamycin (Berg et al., 1975). This

technique has been employed by others to insert a selectable marker into indigenous plasmids in Rhizobium to facilitate the selection of their transfer between different bacteria (Johnston et al., 1978a; Hooykaas et al., 1981). Recently, native markers, i.e. medium bacteriocin-production (Hirsch, 1979) and melanin production (Beynon, Beringer and Johnston, 1980) were found to be associated with transmissible plasmids in R. leguminosarum and R. phaseoli respectively. Such native markers are stable and their exploitation has provided a fruitful start to understanding the role of large plasmids in symbiosis. Nevertheless, the marking technique mentioned above is a necessity, in the absence of recognizable native markers, in the assessment of the genetic functions conferred by indigenous plasmids in Rhizobium (Beringer, Brewin and Johnston, 1980; Brewin et al., 1980a; Johnston and Brewin, 1981):

(i) Conjugative activity (Tra^+):

The plasmid pMAM-4 is transferred from R. trifolii P_3 to R. leguminosarum strain 1056 at a frequency of 10^{-6} per recipient as measured by the frequency of transfer of kanamycin resistance. Resident sex factors in Rhizobium may be rare. In two surveys, three out of 97 isolates of R. leguminosarum (Hirsch, 1979), and one out of 145 isolates of R. phaseoli (Beynon, quoted in Beringer et al., 1980) were shown to carry transmissible bacteriocinogenic plasmids. However, the

frequency of transfer exhibited by pMAM-4 was similar to that of plasmid pRL5JI, an indigenous plasmid in R. leguminosarum strain TOM (Brewin, Beringer and Johnston, 1980b). Other transmissible plasmids, i.e. pRL1JI, pRL3JI and pRL4JI, were found to have a higher frequency of transfer, i.e. 10^{-2} per recipient and to mobilize chromosomal genes at detectable frequencies (10^{-6} to 10^{-7} per recipient) (Hirsch, 1979). Such variability in the transmissibility among Rhizobium plasmids may be attributed to the properties of the donor and the recipient strains as well as to the transfer functions specified by the plasmid itself.

(ii) Host-range specificity (Hsp^+):

One aspect of symbiosis is the specificity whereby particular legume species are nodulated only by certain Rhizobium species; indeed R. trifolii and R. leguminosarum are distinguished by their ability to nodulate clovers and members of Vicieae respectively. However, the transfer of the kanamycin-resistant derivative of pMAM-4 to R. leguminosarum (1056) allowed some of the transconjugant clones, i.e. nine out of 20, to nodulate clover. The resident plasmid in R. trifolii is clearly important as a determinant of host-range specificity since recipient controls did not nodulate clover. Plasmid-mediated transfer of host-range specificity has been reported to occur between different Rhizobium species

(Johnston et al., 1978a; Beynon et al., 1980; Brewin et al., 1980b) and other bacterial genera, e.g. Agrobacterium (Hooykaas et al., 1981). However, nodulation of clover by R. leguminosarum transconjugants was delayed and reduced compared with the control of R. trifolii (MD-1b). These results are consistent with the interpretations of Johnston et al. (1978a) which suggested that the presence in the same Rhizobium strain of genetic information needed to nodulate host plants of different cross-inoculation groups might cause it to be impaired in its nodulation of either host. The analysis of the plasmid content of the clones involved (Beynon et al., 1980) could clarify this observation and give direct evidence for the proposal that there is an interaction between the resident plasmids in Rhizobium. The nodules formed by R. leguminosarum transconjugants on clover were Fix^-. Similarly, variations between different recipient classes and between transconjugants of the same recipient, in their ability to reduce acetylene, have been recorded (Johnston et al., 1978a; Beynon et al., 1980). This indicates that specificity becomes more apparent when the effectiveness of N_2-fixation and not merely nodule-forming ability is considered. Nodulation that occurs outside the common-cross inoculation group, i.e. "abnormal" nodulation, is seldom effective in N_2-fixation (Hepper and Lee, 1979).

(iii) Incompatibility (Inc^+):

The attempt made to define the incompatibility group to which the conjugative plasmid pMAM-4 belongs led to the observation that restriction and modification can occur in R. trifolii strain P_3. The plasmid pMAM-4 appears to be related to the Inc P group plasmids as determined by lysis of the bacteria carrying this plasmid by the donor specific phages PRR1 and PR4 (Bradley, 1976). However, incompatibility has been defined as the inability of two distinct plasmids to be stably co-inherited in a single clone of dividing bacteria in the absence of continued selection pressure for both plasmid types (Timmis, 1979). On the basis of this definition, incompatibility has been demonstrated between some of the resident conjugative plasmids in Rhizobium (Beynon et al., 1980; Brewin et al., 1980b). However, none of the incompatible plasmids was assigned to any of the known incompatibility groups of plasmids (Datta, 1979). As mentioned before, the phages PRR1 and PR4 were restricted by R. trifolii strain P_3. Restriction was of high order, i.e. $\leqslant 10^8$. This points to the likely occurrence of nucleases in the restricting strain that inhibit the transfer of bacterial plasmids particularly in inter-generic transfer attempts (Cunningham, 1979). However, the modified forms of both phages plaqued with equal efficiency on the three hosts, i.e. R. trifolii, E. coli and P. aeruginosa.

Host-controlled or phenotypic modification of phage DNA by some strains of bacteria and the recognition of unmodified DNA by other "restricting" strains has been described for two phages of R. leguminosarum (Schwinghamer, 1965, 1966) and has also been observed in a phage of R. trifolii (Schwinghamer, 1971).

2. Isolation of symbiotic defective mutants in R. trifolii:

(a) Heat treatment:

The elimination of plasmids by growing their hosts at elevated temperatures was first employed by May, Houghton and Perret (1964) with Staphylococcus and by Terawaki, Takayasu and Akiba (1967) with Proteus. The most reasonable explanations for the phenomenon of plasmid-curing following heat treatment are that the plasmid either does not replicate in complete synchrony with chromosomal DNA or is not partitioned efficiently to daughter cells during cell division. Incubation of strain (MD-1a) at 37°C for 48 hrs caused 9% of the clones isolated to have the Nod$^-$ phenotype and 5% to form nodules that were ineffective. The parental strain was shown to harbour two plasmids (see chapter III). The loss of symbiotic properties from R. trifolii (MD-1a) may similarly be due to the temperature-sensitive replication of the resident

plasmid(s) that carry symbiosis genes. Plasmids in Rhizobium are involved in nodulation (Brewin et al., 1980a; Lorkiewicz et al., 1981) and fixation (Ruvkun and Ausubel, 1980a; Kondorosi et al., 1981; Prakash et al., 1981). However, although promising, these results, and subsequently the conclusion, can only be valuable if they are checked rigorously as follows:

(i) It is important from a genetic standpoint, that a marker is introduced into the plasmid, for instance using known transposons (Beringer et al., 1978b). Alternatively, native markers, e.g. cell wall polysaccharide can serve as good tools to indicate the presence or absence of a plasmid (Prakash et al., 1980).

(ii) Physical analysis of the DNA content of both the "cured" clones and the parental strains to clarify the extent of the plasmid loss, whether it is partial, i.e. a deletion or a complete loss (Zurkowski and Lorkiewicz, 1979).

(iii) For complete proof of the role of the plasmid loss in the generation of a defect in the nodulation process or in fixation, the restoration of these functions upon re-introduction of the plasmid is necessary. This will exclude the possibility of a secondary mutation due to heat treatment (Hookyaas et al., 1981).

Variants which have been cured of a plasmid altogether are important in studying the genetics of that plasmid. Heat

treatment has proved efficient in generating symbiotic-defective mutants due to plasmid-curing (Zurkowski, 1981). Curing agents, e.g. acridine orange, have also been used to generate symbiotic-defective mutants in Rhizobium (Higashi, 1967). However, neither heat treatment nor acridine orange is universally successful, and to date there is no report of a strain of Rhizobium that has been cured of all its plasmids.

(b) Tn-5 mutagenesis:

A range of symbiotic-defective mutants of Rhizobium have been produced by classical mutagenic techniques using chemicals or radiation as mutagens (Maier and Brill, 1976; Beringer, Johnston and Wells, 1977). Recently, transposons have been used to induce mutations in a wide range of bacterial species (Kleckner, Roth and Botstein, 1977). The advantages of using transposon mutagenesis with the transposon Tn-5 are that: (1) it induces insertion mutations at single sites; (2) the mutation is labelled by simultaneously acquiring the kanamycin resistance marker encoded by the transposon; (3) the DNA into which the transposon becomes integrated can be isolated by screening fragmented DNA for the piece(s) which hybridize with the transposon (Ruvkin and Ausubel, 1980b).

A total of five transposon-induced symbiotic-defective mutants have been generated using the vector pJB4JI (Beringer

et al., 1978b). In all cases, the defect appeared to affect specifically the ability of the R. trifolii - T. repens symbiosis to fix nitrogen. Whether this was the result of a direct mutation involving the nitrogenase complex or an indirect one, e.g. affecting energy supply within the bacteroid, could not be ascertained. The insertion of Tn-5 was considered to be responsible for the mutant phenotypes observed. Transposon mutagenesis in Rhizobium has facilitated the isolation of specific mutants blocked in steps leading to a functional symbiosis (Buchanan-Wollaston et al., 1980; Ruvkin and Ausubel, 1980b; Rolfe et al., 1981).

(c) Mapping of the transposon-induced symbiotic-defective mutants:

As mentioned before, the mapping of the symbiosis genes after transposon mutagenesis can be done by mapping the drug resistance encoded by the transposon. The insertion sites for Tn-5 in the five isolated mutants of R. trifolii were found to be chromosomally located. Similarly, Cunningham (1979) found that a symbiosis gene in R. trifolii is located chromosomally and Meade et al. (1979) located genes involved in the R. meliloti-alfalfa symbiosis on the bacterial chromosome.

One of the interesting findings during this project was the discovery of multiple sites of insertion for Tn-5 on the chromosome, i.e. 2 - 3 sites in each mutant produced by Tn-5

mutagenesis. Such genetic instability has been shown by the transposon Tn-1816, a transposon coding for mercury resistance, leading to multiple auxotrophy in P. putida and E. coli (Friello and Chakrabarty, 1980). Furthermore, introductions of RP4 into strains harbouring Tn-1816 and subsequent restriction endonuclease analysis of RP4::Tn-1816 revealed that multiple copies of Tn-1816 occurred on the RP4 plasmid. Similar behaviour has been shown by the transposon Tn-501, another transposon determining resistance to mercury (Bennett et al., 1978). Various attempts have been made to explain non-homologous recombination events in transposition by a systematic model (Arthur and Sherratt, 1979; Grindley and Sherratt, 1979; Shapiro, 1979; Read, Das Sarma and Jaskunas, 1980; Galas and Chandler, 1981; Harshey and Bukhari, 1981). The following features seem to be applicable to most, if not all, models of DNA transposition: (1) replication of the transposable element and conservation or regeneration of the donor site; (2) specific DNA cuts at the termini of the transposable element; (3) staggered DNA cuts at the target site; (4) generation of replicon fusion structures as transposition intermediates or products of a process closely related to transposition (Shapiro, 1980; Bukhari, 1981). However, the results in this thesis do not permit the choice unequivocally of one model from the various models described in recent

literature. The best that can be achieved is to eliminate the simple model of Berg (1977). In this model, a phage lambda-like excision-integration system for Tn-5 operates, in which the element possesses a mechanism whereby it is precisely excised, at a high frequency, and reinserted at another site with the subsequent exonucleolytic degradation of the donor molecule.

The creation of multiple transposon insertions in the chromosome in all the clones investigated precludes the use of Tn-5 mutagenesis as a method of investigating the chromosomal location of individual symbiosis genes.

3. COMPETITION IN RHIZOBIUM

The present investigation used mutation to antibiotic resistance as a marker technique, which has the distinct advantage over serological methods of being both rapid and sensitive. Such genetic markers are stable and different strains may be labelled with ease. The results demonstrated the usefulness of this technique in studies of competition between strains in nodule formation especially in identifying mixed-infected nodules. In some cases, the frequency of one of the strains was less than 0.1% of the total number of viable bacteria isolated from the nodule, yet this strain was readily detected. However, the main disadvantage is that mutant strains are being used in place of wild-type strains.

Mutation to antibiotic resistance can be associated with partial loss of effectiveness in nitrogen fixation (Jones and Bromfield, 1978; Pain, 1979). However, the results reported in the above-cited references showed that it is only a minority of the mutants isolated that had a modified symbiotic phenotype and by screening adequate numbers of mutants it would be possible to select strains having effectiveness indistinguishable from the parental strains. Recently, Josey et al. (1979) used the intrinsic low-level resistance to a range of antibiotics to characterize wild-type and genetically marked strains of Rhizobium. This test, though it has the advantage that it does not demand alterations in the strain which may interfere with its symbiotic performance, does not give optimal reproducibility over an extended period and because of this instability, accurate strain identification could not be made without reference to a control culture. However, it is possible that other intrinsic properties such as phage-resistance and bacteriocin production may be applicable to the problem of strain identification in Rhizobium.

There were marked differences in competitiveness as measured by success in nodulation, between the mutant strains used. Surprisingly, the United Kingdom strains (MD-1b and MD-1c) were more competitive at 25^{o}C than the Iraqi strain (MD-1a). This may be attributed to specific host effects in determining the relative success in nodulation by the competing strains, since the host variety

from which the Iraqi strain (MD-1a) was originally isolated, and was therefore compatible with, is Trifolium alexandrium and not T. repens. Similar selection by clover varieties of specific strains of nodule bacteria from a mixture present in the rhizosphere has been observed (Russel and Jones, 1975; Jones and Hardarson, 1979). However, the results of this investigation indicated that the competitive ability of a strain, which may be affected by host preference, can be markedly altered by temperature as can be seen from the increase in the frequency of both single and double strain occupancy of strain (MD-1a) at 25°C compared with its performance at 15°C. This confirms results published by Hardarson and Jones (1979) and emphasises the necessity for information on environmental factors, such as soil temperature, to establish a compatible relationship between a legume variety and an effective strain of Rhizobium.

In contrast with legumes grown in soil (Bromfield and Jones, 1980), the results showed that the frequency of doubly infected nodules is high (33.3% in the combination MD-1c + MD-1b). Such a high frequency of mixed strain occupancy has been observed previously (Johnston and Beringer, 1975; Bromfield and Jones, 1980) when legumes are grown under laboratory conditions. This artefact may be explained by the fact that the growth of aseptic plants under conditions where the two bacterial strains are present in a continuous film of moisture on the root surface is conducive to

double invasion. However, in mixed-infected nodules, strain (MD-1c) formed the majority of the population in all combinations and at both temperatures. This may add another factor for its superiority as a competitor and could be explained by any of the following:

1. Strain (MD-1c) is more competitive in the formation of infection threads, although the data obtained do not clarify whether there is a mixture of bacterial strains within a single infection thread, or whether two separate threads, each containing a single genotype, are involved. In any case, if the nodule is formed by the progeny of a single bacterium, there is no exclusion mechanism against subsequent invasion by other bacteria.

2. Since the competing strains have a similar growth rate, in so far as they produced similar sized colonies in three days, strain (MD-1c) could be more competitive in the utilization of carbon sources in the nodule. This could be confirmed by biochemical analysis of the competing strains separately.

3. Strain (MD-1c) could be bacteriocinogenic or lysogenic. Such properties would alter the proportions of strains of rhizobia growing in a mixture.

However, the relevance of this study to the field situation is not clear. Johnson, Means and Weber (1965) found that one strain of R. japonicum which was competitive in the greenhouse was not competitive in the field. Other strains were competitive under both

regimes.

It is likely that, in field situations, the degree of competitiveness is influenced by other parameters such as the ability to survive and multiply in the presence of indigenous microbial species absent from the greenhouse trials. The need to analyse the competitiveness of Rhizobium arises from the desire to promote nodulation specifically using a particular inoculant strain. Brewin, Johnston and Beringer (1980) suggested a strategy to ensure this specific interaction by using a host genotype with an extremely rigorous specificity for nodulating rhizobia. This approach, although very limited, leading to the accumulation of knowledge concerning the factors affecting only one particular liaison would, nevertheless, provide a good basis for the exploitation of competition.

To understand the factors involved in the successful liaison it would be necessary to analyse both genetic and environmental factors. The genetic factors would include the involvement of the indigenous conjugative plasmids of Rhizobium in nodulation, fixation and bacteriocinogeny. The environmental factors, important as they are in ultimate practice, are too complex to readily permit separate evaluation. Nevertheless, an attempt has to be made to unravel the various effects of the environment on competition of Rhizobium in natural situations. Competition between Rhizobium strains can take place during the stages leading to successful nodulation. It would be necessary to assess the time

required for each stage to be completed in order to determine the sensitivity of each to alteration in the environment. Thus, Munns (1968) found that prevention of nodulation by acidity in heavily inoculated solution cultures could be attributed to prevention of a step which approximately coincides temporally with the curling of root hairs. This step occupied less than 12 hours of the 4 to 7 days required for visible nodules to appear. Moreover, Winaro and Lie (1979) showed that the critical period of competition is restricted to 24 hrs after inoculation.

For a completely coherent picture of competition, greenhouse trials would be used to forecast the outcome of field trials, the latter being the ultimate test of a strain's ability to compete, since the proportion of nodules formed and the amount of nitrogen fixed are the end result of many interacting factors.

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"APPENDIX"

Table 1: Nodule-strain identity of the combination (MD-1b) plus (MD-1c) at 20°C

No. of nodule	(MD-1b)	(MD-1c)	No. of nodule	(MD-1b)	(MD-1c)
1	9.5×10^2	2.1×10^4	13	-	6.1×10^4
2	1.0×10^2	1.6×10^3	14	2.1×10^4	-
3	90	2.2×10^4	15	1.0×10^2	-
4	-	1.9×10^3	16	-	2.4×10^4
5	4.4×10^2	7.2×10^3	17	1.0×10^2	3.1×10^3
6	1.2×10^2	3.2×10^4	18	-	2.9×10^4
7	1.8×10^2	2.6×10^4	19	-	1.0×10^4
8	4.5×10^2	-	20	4.8×10^2	-
9	1.4×10^4	-	21	-	2.0×10^4
10	-	3.2×10^4	22	-	8.8×10^2
11	3.0×10^2	-	23	2.2×10^2	3.6×10^4
12	1.15×10^2	-	24	30	-

Table 1 (contd.)

No. of nodule	(MD-1b)	(MD-1c)	No. of nodule	(MD-1b)	(MD-1c)
25	1.3×10^3	-	37	25	2.3×10^4
26	-	9.8×10^4	38	25	4.6×10^4
27	-	5.7×10^4	39	50	7.8×10^3
28	20	5.4×10^4	40	-	6.9×10^4
29	-	6.9×10^3	41	2.2×10^3	-
30	45	2.7×10^3	42	-	4.1×10^4
31	-	6.6×10^4	43	-	1.8×10^4
32	3.1×10^4	-	44	1.2×10^2	4.9×10^4
33	20	2.3×10^2	45	4.6×10^2	1.0×10^2
34	-	2.0×10^4	46	75	7.2×10^4
35	15	3.1×10^4	47	1.5×10^2	9.8×10^4
36	35	6.6×10^4	48	2.4×10^4	-

Table 1 (contd.)

No. of nodule	(MD-1b)	(MD-1c)	No. of nodule	(MD-1b)	(MD-1c)
49	1.2×10^2	9.7×10^4	61	2.9×10^3	-
50	-	1.9×10^4	62	4.4×10^2	-
51	3.5×10^2	-	63	95	-
52	1.7×10^2	-	64	1.5×10^2	1.2×10^3
53	1.6×10^2	2.3×10^4	65	1.7×10^2	-
54	40	7.5×10^4	66	-	3.4×10^4
55	1.3×10^3	-	67	7.0×10^2	-
56	-	7.1×10^4	68	-	1.8×10^3
57	2.3×10^2	-	69	2.9×10^2	-
58	2.4×10^2	-	70	1.1×10^2	3.8×10^4
59	1.3×10^3	-	71	1.1×10^2	5.5×10^4
60	5.4×10^2	-	72	1.5×10^2	6.2×10^4

Table 1 (contd.)

No. of nodule	(MD-1b)	(MD-1c)	No. of nodule	(MD-1b)	(MD-1c)
73	3.2×10^2	5.6×10^4	85	9.8×10^2	4.0×10^4
74	-	1.7×10^4	86	7.9×10^2	3.2×10^3
75	-	1.1×10^4	87	-	6.8×10^4
76	-	2.0×10^4	88	56	1.1×10^4
77	85	2.7×10^4	89	20	2.7×10^4
78	1.5×10^3	-	90	-	2.9×10^3
79	1.2×10^2	1.5×10^3	91	2.4×10^2	-
80	50	9.7×10^4	92	-	5.1×10^2
81	20	1.4×10^4	93	35	-
82	-	3.7×10^4	94	1.1×10^2	1.7×10^4
83	-	7.6×10^3	95	5.9×10^2	-
84	3.6×10^2	9.2×10^3	96	3.8×10^2	-

Table 1 (contd.)

No. of nodule	(MD-1b)	(MD-1c)	No. of nodule	(MD-1b)	(MD-1c)
97	3.1×10^2	5.2×10^4	109	20	-
98	1.3×10^3	-	110	-	1.6×10^2
99	75	-	111	-	5.6×10^4
100	25	-	112	-	5.6×10^4
101	20	-	113	4.0×10^2	-
102	-	3.4×10^4	114	-	6.3×10^4
103	-	2.6×10^2	115	1.4×10^4	-
104	15	-	116	-	3.6×10^3
105	85	8.6×10^3	117	2.2×10^2	-
106	-	1.3×10^2	118	-	5.4×10^4
107	-	7.2×10^3	119	-	4.8×10^4
108	40	-	120	-	3.2×10^3

(4)

Table 2: Nodule-strain identity of the combination (MD-1b) plus (MD-1a) at 25°C

No. of nodule	(MD-1b)	(MD-1a)	No. of nodule	(MD-1b)	(MD-1a)
1	2.0×10^3	-	13	2.8×10^4	-
2	1.0×10^4	25	14	85	3.2×10^3
3	2.5×10^4	-	15	2.9×10^4	-
4	2.6×10^4	15	16	45	-
5	1.2×10^3	-	17	7.5×10^3	-
6	-	4.2×10^3	18	2.5×10^2	-
7	1.4×10^4	-	19	2.1×10^4	-
8	7.1×10^3	1.0×10^4	20	2.7×10^4	-
9	45	-	21	4.6×10^3	-
10	6.7×10^2	-	22	9.1×10^2	-
11	2.5×10^4	-	23	9.1×10^3	1.0×10^4
12	6.3×10^2	-	24	6.3×10^2	-

Table 2 (contd.)

No. of nodule	(MD-1b)	(MD-1a)	No. of nodule	(MD-1b)	(MD-1a)
25	9.2×10^3	-	37	3.2×10^4	-
26	2.0×10^3	-	38	2.5×10^3	1.0×10^2
27	2.1×10^4	-	39	1.1×10^2	-
28	30	-	40	7.4×10^2	-
29	1.4×10^3	-	41	2.4×10^4	5.1×10^3
30	-	50	42	1.9×10^3	25
31	5.0×10^2	-	43	5.6×10^3	-
32	7.0×10^3	2.4×10^3	44	45	-
33	-	2.7×10^4	45	6.5×10^3	-
34	3.8×10^2	-	46	4.7×10^3	-
35	1.0×10^2	-	47	-	3.1×10^4
36	4.5×10^2	-	48	-	2.2×10^4

Table 2 (contd.)

No. of nodule	(MD-1b)	(MD-1a)	No. of nodule	(MD-1b)	(MD-1a)
49	1.0×10^2	-	61	9.4×10^2	-
50	65	-	62	95	-
51	1.7×10^4	2.3×10^4	63	2.4×10^2	-
52	-	30	64	1.1×10^4	-
53	5.0×10^3	-	65	5.0×10^2	-
54	-	2.7×10^4	66	8.1×10^2	-
55	-	1.7×10^2	67	-	1.2×10^4
56	-	2.9×10^4	68	1.0×10^4	-
57	9.4×10^2	-	69	5.6×10^3	-
58	1.9×10^2	-	70	-	40
59	3.5×10^2	55	71	3.1×10^2	-
60	6.0×10^2	-	72	5.7×10^2	-

Table 2 (contd.)

No. of nodule	(MD-1b)	(MD-1a)	No. of nodule	(MD-1b)	(MD-1a)
73	3.8×10^2	-	79	8.1×10^2	-
74	7.9×10^3	-	80	2.2×10^2	-
75	1.7×10^4	4.7×10^3	81	1.1×10^3	-
76	6.6×10^2	-	82	2.9×10^2	70
77	2.2×10^3	1.2×10^2	83	86	-
78	1.1×10^3	8.8×10^3	84	7.0×10^2	-

Table 3: Nodule-strain identity of the combination (MD-1b) plus (MD-1a) at $15^{o}C$

No. of nodule	(MD-1b)	(MD-1a)	No. of nodule	(MD-1b)	(MD-1a)
1	1.3×10^2	-	13	5.3×10^3	-
2	6.0×10^2	-	14	2.2×10^2	-
3	1.0×10^2	-	15	8.3×10^2	-
4	2.1×10^2	-	16	1.5×10^2	-
5	3.3×10^3	1.5×10^3	17	6.2×10^2	-
6	35	-	18	2.6×10^2	-
7	9.3×10^3	-	19	2.3×10^3	-
8	1.2×10^3	-	20	1.3×10^3	-
9	6.2×10^2	-	21	3.4×10^2	-
10	1.0×10^2	-	22	2.4×10^2	-
11	4.0×10^2	-	23	4.5×10^2	-
12	1.0×10^4	-	24	3.9×10^2	-

(x)

Table 3 (contd.)

No. of nodule	(MD-1b)	(MD-1a)	No. of nodule	(MD-1b)	(MD-1a)
25	7.2×10^2	-	37	2.2×10^2	-
26	1.0×10^3	-	38	2.0×10^3	-
27	2.2×10^2	-	39	1.2×10^2	-
28	4.1×10^2	-	40	6.5×10^3	-
29	2.1×10^2	-	41	9.1×10^2	3.5×10^2
30	1.0×10^3	-	42	1.1×10^3	-
31	45	-	43	2.3×10^3	-
32	1.4×10^3	-	44	4.7×10^2	1.1×10^2
33	1.0×10^3	-	45	1.1×10^3	-
34	2.3×10^3	2.5×10^2	46	9.2×10^2	-
35	35	-	47	3.2×10^2	-
36	1.0×10^3	-	48	2.1×10^2	-

Table 3 (contd.)

No. of nodule	(MD-1b)	(MD-1a)	No. of nodule	(MD-1b)	(MD-1a)
49	1.8×10^4	-	61	2.1×10^3	-
50	2.0×10^2	-	62	2.2×10^3	-
51	3.6×10^2	-	63	2.4×10^3	-
52	65	15	64	90	-
53	95	-	65	7.8×10^2	-
54	-	2.5×10^2	66	2.1×10^2	-
55	1.3×10^2	-	67	5.4×10^2	-
56	1.1×10^2	-	68	1.6×10^2	-
57	55	-	69	3.4×10^3	-
58	1.2×10^2	-	70	1.9×10^2	-
59	4.6×10^3	-	71	2.9×10^2	-
60	2.0×10^3	-	72	3.6×10^2	-

Table 3 (contd.)

No. of nodule	(MD-1b)	(MD-1a)	No. of nodule	(MD-1b)	(MD-1a)
73	1.2×10^3	-	79	1.4×10^2	-
74	1.4×10^2	-	80	6.5×10^2	-
75	2.8×10^2	-	81	3.2×10^2	-
76	7.2×10^2	-	82	3.6×10^2	-
77	2.9×10^3	-	83	1.0×10^3	-
78	2.8×10^2	-	84	2.9×10^2	-

Table 4: Nodule-strain identity of the combination (MD-1c) plus (MD-1a) at 25°C

No. of nodule	(MD-1c)	(MD-1a)	No. of nodule	(MD-1c)	(MD-1a)
1	-	1.0×10^4	13	1.8×10^4	-
2	1.3×10^3	-	14	45	-
3	-	6.8×10^2	15	1.0×10^2	85
4	2.0×10^4	-	16	2.6×10^3	-
5	9.2×10^4	-	17	1.0×10^4	-
6	4.9×10^4	1.0×10^3	18	7.1×10^2	-
7	1.2×10^3	-	19	3.2×10^4	2.2×10^4
8	5.4×10^3	-	20	1.2×10^3	-
9	2.8×10^2	-	21	6.8×10^4	-
10	2.0×10^2	-	22	6.0×10^4	1.2×10^3
11	-	7.0×10^2	23	6.3×10^4	-
12	1.0×10^3	-	24	3.0×10^2	-

Table 4 (contd.)

No. of nodule	(MD-1c)	(MD-1a)	No. of nodule	(MD-1c)	(MD-1a)
25	1.9×10^4	-	37	7.7×10^3	25
26	1.2×10^3	-	38	1.0×10^4	-
27	3.3×10^4	-	39	2.4×10^4	-
28	1.6×10^3	6.4×10^2	40	1.8×10^4	2.8×10^3
29	6.6×10^4	-	41	2.0×10^3	-
30	1.0×10^5	-	42	1.2×10^3	-
31	1.0×10^6	-	43	4.2×10^3	-
32	3.6×10^4	-	44	8.5×10^3	-
33	1.7×10^4	-	45	3.9×10^2	-
34	8.3×10^3	-	46	-	35
35	1.4×10^4	-	47	1.0×10^4	-
36	4.9×10^3	-	48	1.4×10^3	-

Table 4 (contd.)

No. of nodule	(MD-1c)	(MD-1a)	No. of nodule	(MD-1c)	(MD-1a)
49	1.3×10^3	-	61	1.7×10^4	-
50	4.5×10^4	1.3×10^2	62	2.5×10^3	-
51	2.8×10^3	-	63	1.0×10^4	1.8×10^2
52	-	1.7×10^3	64	5.4×10^3	-
53	6.6×10^3	1.2×10^2	65	4.3×10^4	95
54	4.1×10^2	-	66	4.9×10^3	50
55	2.6×10^4	-	67	1.9×10^3	-
56	2.4×10^4	-	68	5.4×10^3	-
57	2.7×10^3	-	69	2.1×10^4	-
58	2.7×10^4	-	70	2.0×10^3	-
59	4.4×10^4	-	71	9.4×10^3	35
60	-	3.6×10^2	72	1.0×10^3	-

Table 4 (contd.)

No. of nodule	(MD-1c)	(MD-1a)	No. of nodule	(MD-1c)	(MD-1a)
73	4.8×10^3	-	84	1.0×10^4	-
74	5.5×10^3	-	85	50	-
75	4.3×10^4	30	86	6.1×10^2	-
76	6.7×10^3	-	87	2.1×10^4	-
77	9.7×10^3	-	88	1.0×10^4	-
78	2.3×10^4	-	89	3.0×10^2	-
79	6.5×10^3	-	90	1.5×10^3	-
80	3.3×10^2	-	91	1.0×10^3	-
81	1.9×10^4	-	92	2.3×10^4	-
82	2.1×10^4	35	93	8.5×10^3	-
83	3.3×10^4	-	94	1.1×10^2	-

Table 5: Nodule-strain identity of the combination (MD-1c) plus (MD-1a) at 15°C

No. of nodule	(MD-1c)	(MD-1a)	No. of nodule	(MD-1c)	(MD-1a)
1	2.9×10^3	-	13	2.0×10^4	-
2	2.1×10^3	-	14	1.0×10^3	-
3	1.5×10^4	-	15	3.4×10^3	-
4	1.4×10^4	-	16	1.5×10^2	-
5	2.7×10^3	-	17	1.2×10^3	-
6	2.1×10^3	-	18	6.6×10^3	-
7	1.9×10^3	-	19	6.9×10^2	-
8	5.4×10^3	-	20	2.8×10^3	-
9	1.6×10^3	-	21	1.6×10^3	-
10	1.2×10^3	-	22	5.5×10^3	-
11	1.7×10^3	45	23	7.8×10^3	-
12	1.9×10^3	-	24	2.3×10^4	-

Table 5 (contd.)

No. of nodule	(MD-1c)	(MD-1a)	No. of nodule	(MD-1c)	(MD-1a)
25	5.5×10^2	-	37	1.4×10^4	-
26	6.3×10^2	15	38	1.7×10^4	-
27	1.8×10^3	-	39	1.0×10^5	-
28	2.7×10^4	-	40	1.4×10^3	-
29	1.9×10^4	-	41	1.8×10^4	-
30	4.5×10^3	-	42	1.4×10^3	-
31	2.2×10^2	-	43	1.9×10^4	-
32	1.5×10^4	-	44	2.6×10^4	-
33	1.4×10^3	-	45	2.4×10^4	-
34	3.5×10^2	-	46	1.0×10^3	-
35	6.0×10^3	-	47	1.0×10^3	-
36	8.3×10^3	-	48	3.0×10^4	-

Table 5 (contd.)

No. of nodule	(MD-1c)	(MD-1a)	No. of nodule	(MD-1c)	(MD-1a)
49	8.9×10^2	-	61	3.2×10^4	-
50	2.5×10^4	-	62	5.2×10^3	-
51	3.1×10^4	-	63	6.4×10^3	-
52	3.8×10^4	45	64	2.4×10^2	-
53	2.4×10^4	-	65	3.1×10^4	-
54	1.2×10^4	-	66	3.5×10^4	-
55	1.5×10^3	-	67	3.4×10^4	-
56	9.2×10^3	-	68	3.3×10^4	-
57	2.1×10^2	-	69	3.6×10^4	-
58	1.6×10^4	-	70	1.5×10^4	-
59	3.2×10^4	1.8×10^2	71	1.2×10^3	-
60	3.8×10^4	-	72	2.2×10^3	15

Table 5 (contd.)

No. of nodule	(MD-1c)	(MD-1a)	No. of nodule	(MD-1c)	(MD-1a)
73	3.1×10^4	-	85	6.1×10^2	-
74	1.5×10^4	-	86	2.0×10^4	-
75	2.5×10^4	-	87	1.0×10^4	-
76	6.1×10^2	-	88	30	-
77	1.2×10^4	-	89	7.5×10^2	-
78	2.8×10^4	3.5×10^2	90	4.5×10^2	-
79	2.2×10^4	-	91	8.0×10^3	-
80	6.0×10^2	-	92	1.1×10^3	25
81	1.9×10^3	-	93	4.1×10^2	-
82	2.0×10^2	-	94	1.2×10^4	-
83	45	-	95	1.0×10^3	-
84	4.5×10^2	-	96	3.3×10^2	-

Table 5 (contd.)

No. of nodule	(MD-1c)	(MD-1a)	No. of nodule	(MD-1c)	MD-1a)
97	5.6×10^3	-	100	1.1×10^2	-
98	4.8×10^3	-	101	2.7×10^3	-
99	1.2×10^4	-			